ABNORMAL MORPHOGENESIS OF SKIN AND FEATHERS IN THE CHICKEN EMBRYO INDUCED BY THE L-PROLINE ANALOG, L-AZETIDINE-2-CARBOXYLIC ACID: A LIGHT MICROSCOPICAL, ULTRASTRUCTURAL (SEM AND TEM) AND CHEMICAL ANALYSIS

A DISSERTATION

SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

ANDREA LEWIS MILLER

DEPARTMENT OF BIOLOGY

ATLANTA, GEORGIA

AUGUST 1980

DOCTOR OF PHILOSOPHY DISSERTATION

OF

ANDREA LEWIS MILLER

Approved:

Major Professor: _____ Date: 7/10/80

Dissertation Committee Member: _____

Dissertation Committee Member: _____

Department Chairman: _____

Dean, School of Arts and Sciences: _____

ABSTRACT

BIOLOGY

MILLER, ANDREA LEWIS B.S., LeMOYNE-OWEN COLLEGE, 1976

M.S., ATLANTA UNIVERSITY, 1978

ABNORMAL MORPHOGENESIS OF SKIN AND FEATHERS IN THE CHICKEN EMBRYO INDUCED BY THE L-PROLINE ANALOG, L-AZETIDINE-2-CARBOXYLIC ACID: A LIGHT MICROSCOPICAL, ULTRASTRUCTURAL (SEM AND TEM) AND CHEMICAL ANALYSIS.

Advisor: Dr. Roy Hunter Jr.

Doctor of Philosophy degree conferred August 1, 1980

Dissertation dated August 1980

Collagen is known to play a vital role in several developing systems which depend on epithelio-mesenchymal interactions for their normal morphogenesis. The development of embryonic chick skin has been found to be dependent on interactions between mesoderm (dermis) and ectoderm (epidermis) and collagen has been implicated in the formation of feather germs and feather patterns. Since collagen is the most abundant protein found in connective tissue and the major structural constituent of skin, the problem of whether normal skin morphogenesis is dependent on normal collagen biosynthesis is the subject of concern.

The use of several proline analogs has provided a useful tool in exploring the morphogenesis of several differentiating collagen-dependent systems. In vitro studies have shown that the structural

proline analog, L-azetidine-2-carboxylic (LACA), interferes with the normal biosynthesis of collagen molecules. The procollagen polypeptide chains containing the analog are underhydroxylated and, therefore, do not fold into the normal triple helical conformation; this causes a delay in the secretion of the molecule into the extracellular matrix. The purpose of this investigation was, therefore, to study the abnormal morphogenetic and biochemical changes that occurred in chick embryo skin due to the administration of the proline analog, LACA, and to determine if these changes were due possibly to an interruption of collagenogenesis.

Fertile eggs of the White Leghorn Variety chicken were injected with 0.6 cc of 1 mg/ml of solution of LACA through the air sacs at pre-incubation, 24 hr and at 48 hr of incubation. Single injections of 0.9 cc of 1 mg/ml of LACA solution were also given at pre-incubation, 16-18 hr, 24 hr, or 48 hr of incubation. The embryos were removed from their shells and analyzed for suppression and/or inhibition of feathers. The time at which the single injections were made proved to be critical. The highest percentage of feather suppression (30%) occurred in embryos that were injected at 16-18 hr of incubation. However, triple-injections caused an even greater percentage of feather suppressions and/or inhibitions (35%). This high percentage of feather suppressions was observed in embryos at days 11-12 as opposed to earlier or later days.

Skin whole mounts showed that the LACA-treated embryos did not exhibit the normal pattern of feather distribution. Light microscopical studies showed that in the LACA-treated embryo, there was a decrease in the compactness of dermal condensations of feather germs and a lack of basement membrane formation at the epidermal-dermal junction. Scanning electron microscopy (SEM) of skin from LACA-treated embryos showed a lack of distinct extracellular fibrils in the dermis and "epidermal blistering" of the peridermal sheath in apterylar regions. Transmission electron microscopy (TEM) of skin from LACA-treated embryos revealed (1) a decrease in the number of fibrils in the epidermal-dermal junction (2) noticeable decrease in the number of fibrils secreted by dermal fibroblasts, and (3) an unorganized lattice of collagen fibrils in the intercellular spaces. Hydroxyproline analysis of skin from LACA-treated embryos indicated that the hydroxyproline content of these embryos decreased upon increased amounts of LACA (controls - 5.1 ug/gm skin; single injected LACA-treated - 2.9 ug/gm skin; triple injected LACA-treated - 1.1 ug/gm skin).

It is apparent from these studies that LACA causes malformations in the skin of chick embryos, possibly by interfering with the normal rate of synthesis of extrusion of the collagen molecule. The exact mechanisms by which the feather suppression occurred is not known. However, the proposal is offered that the proline analog altered the normal structure of the collagen molecule which subsequently

decreased the rate of collagen extrusion and thus decreased its synthesis through negative feed-back inhibition, thereby causing suppression and/or inhibition of normal skin and feather development. This was mediated through the lack of normal basement membrane formation.

ACKNOWLEDGEMENTS

Special thanks to God who has given me strength and brought me thus far.

Appreciation is extended to my husband whose love, understanding, encouragement, and unending support made possible the completion of this goal.

Gratitude is extended to my Mom, Dad, and Grandfather whose prayers and concern have given me faith; for them this goal was undertaken.

I am very grateful to my major professor, Dr. Roy Hunter, Jr., under whose strict guidance, meaningful comments, constructive criticisms, and scholarly suggestions this work was achieved; and to his wife, Julie, one of the most beautiful persons whom I have ever met. Thanks are expressed to Dr. J.K. Haynes and Dr. J.E. Mayfield my committee members; your guidance has been invaluable. A portion of this work has been supported by the National Institute of General Medical Sciences, NIH-MARC Grant #1 T02 GM 05007-01.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Collagen is known to play a vital role in several developing systems which depend on epithelial-mesenchymal interactions for their normal morphogenesis. In such situations, it has been proposed that the epithelium contributes to the polymerization of the collagen which is synthesized by the mesenchyme (Kallman and Grobstein, 1965). Bernfield (1970) has shown that the ectoderm (epithelial) also affects the amount of collagen synthesized by the mesoderm. The postulate of a morphogenetic role of collagen is based on the observation of structural alterations in salivary gland (Grobstein and Cohen, 1965), ureteric and lung rudiments (Wessells and Cohen, 1968) and in skin (Stuart and Moscona, 1967) after several experiments involving collagenase.

The development of embryonic skin has been found to be dependent on interactions between the mesoderm and ectoderm (Sengel, 1958; Wessells, 1962; Rawles, 1953) and collagen has been implicated in the formation of feather germs and feather patterns (Stuart and Moscona, 1967). According to their investigations, the establishment of dermal condensations is preceded by the formation of a birefringent fibrous lattice in the mesoderm. Based on the susceptibility of this highly structural lattice to collagenase, its resistance to trypsin and its affinity for Van Gieson's stain (a stain used to demonstrate collagen),

it has been proposed that the lattice is made of collagen (Stuart and Moscona, 1967). The site at which the fibers intersect one another corresponds to the location of the dermal condensations. These structures are thus laid down in a very precise pattern which is the forerunner of the distribution of adult feathers within individual tracts.

The use of several proline analogs has provided information to demonstrate that if folding of collagen pro alpha chains into the triple-helical conformation is prevented, secretion of the protein is delayed (Uitto and Prockop, 1974a; Oikarinen et al., 1976a). These investigators suggested that the incorporation of proline analogs yields pro alpha chains that are normal in size and become disulfide linked but not triple helical. Supposedly, because the pro alpha chains containing the analogs remain non-helical, they are forced to react with hydroxylating and glycosylating enzymes for an extended period.

In chick embryos that are treated with the L-proline analog, L-azetidine-2-carboxylic acid (LACA), the feathers are either inhibited or suppressed and the abnormality has been traced to a failure of the treated embryos' skin to form normal dermal condensations (Lewis and Hunter, 1978). In view of the possible role of the fibrous lattice in the formation of dermal condensations in normal skin, an electron microscopic examination of the deposition of collagen fibers in the extracellular matrix between dermal cells as well as the basement lamina of the control and LACA-treated skin was performed. Phase

contrast microscopy of skin whole mounts and scanning electron microscopy were also utilized in order to study the development of ordered patterns of papillae. This examination extended to skin taken from normal and LACA-treated embryos. One observation previously reported (Lane et al., 1971) is that hydroxyproline is relatively unique to the collagen molecule and that the collagen of analog-treated connective tissues is underhydroxylated. In view of this observation, microgram amounts of hydroxyproline were determined in skin of both normal and LACA-treated embryos as an index of the accumulation of collagen.

CHAPTER II

REVIEW OF LITERATURE

The skin is made of two tissues of distinct origin: the dermis and epidermis. The mechanisms of differentiation of the skin and the cutaneous appendages are dependent on each of these two constituents in the course of embryonic development (Wessells, 1965). In the chick, the feather is one of the primary differentiations of the epidermis (reviewed by Brothman, 1977). It is the expansion of the embryonic feather, the feather germ, which appears in the embryo on the seventh day of incubation.

To review the steps of differentiation of the skin and of the feather germs in the chick embryo, development proceeds as follows:

(1) On day five of incubation, the back of the embryo is covered with a flat undifferentiated epidermis which is composed of two cell layers. The basal layer is composed of cubodial cells, and a superficial one is composed of flattened cells, fewer in number than the basal ones.

(2) On the sixth day of incubation, the epidermis differentiates and acquires its typical structure: a basal layer made of erect cylindrical cells and a superficial layer, the periderm, composed of closely adhering cells.

(3) Until the seventh day of incubation, the dorsal epidermis remains flat and smooth. The first transformations which precede the

differentiation of feather germs take place in the dermis. Toward the middle of the seventh day of incubation, small condensations of dermal cells form on the middorsal line of the embryo. Over each of these masses of dermal cells, the epidermis starts to thicken; this is the stage of the feather primordium. Later, new primordia appear in successive rows on each side of the middorsal line. In the meantime, the epidermis of the primordia of the middorsal line, and later the ones of the dorsolateral rows begin to grow out and form the epidermal sheath of the young feather germs.

(4) On the eighth day, the most advanced germs have taken the form of little cylindrical papillae that protrude above the surface of the skin. They have a well defined orientation in relation to the embryo, being all inclined toward the rear of the embryo.

(5) After the eighth and ninth day of incubation, when dermal fibroblasts begin to synthesize fibrous material, a structure called the basal lamina begins to form. This structure is a sheet of extracellular macromolecules that is located at the interface of epidermis and dermis (Kallman et al., 1967a,b).

The definitive role of the basement membrane complex adjacent to epithelial cells in chick skin has not been established. Speculation based on its position and properties of permeability to molecules of various sizes (Lowenstein and Kang, 1964) has led to the idea that the basement lamina is an important factor in the mediation of extrinsic cues to developing epithelial cells. Wessells (1977) suggests that contact of epidermal cells with the basal lamina maintains mitotic

capacity and inhibits differentiation. Such cells retain competence to respond to a protein called Epithelial Growth Factor (EGF) or other growth factors that stimulate mitotic activity in embryonic epidermis and other developing epithelium, thereby regulating normal thickness of epidermis. Once cells move outward, away from contact with the basal lamina and dermis, they lose that competence, cease dividing, and differentiate to form the mature feather.

A variety of other observations suggest that intercellular relations are involved in the formation of feathers on the body of a chicken embryo. As a result of specific physical factors, the axial system evokes formation of the first row of feather germs along the dorsal midline of the back (Maderson, 1965; for discussion, Wessells, 1967a, b). Afterwards, additional rows arise in a spatial and temporal sequence on each side of the central row. During his studies on feathers, Sengel (1958) noted that oriented mesodermal cells stretch between the dermal condensations of the germs. Stuart and Moscona (1967) have also reported such oriented cells and that collagen may be oriented in a similar pattern. In light of the general supposition that cell orientation may be dependent upon substrate orientation (Weiss, 1961), these observations raise the possibility that extracellular materials may play an influential role in formation of feather germs or of the pattern of such germs on the embryo. As dermal condensations form, peculiar bundles of filaments are seen to extend downward from the epidermal cells situated over the center of each condensation (Wessells, 1965). These bundles are composed of

large numbers of filaments that appear aperiodic after phosphotungstic acid staining (Kallman et al., 1967a). In the course of examining cross sections of these anchor filaments, Wessells and Evans (1968) noted the distribution of collagen fibers in the dermis. Collagen is found in the spaces between dermal condensations and the organization of this fibrous material in the dermis into detectable traits and lattices of aligned fibrils precedes the appearance of the dermal papillae and this foretells incipient dermal morphogenesis.

Collagen is the commonest and most abundant protein in the animal kingdom; it provides an extracellular framework for all multicellular animals (Gross, 1973). Even though collagen is most abundant in fibrous connective tissue, it appears in some shape or form in virtually every tissue. It provides the (1) ropes and straps (tendons and ligaments), (2) woven sheets (skin and fascia), (3) filtration membranes (glomeruli), (4) supporting skeleton reinforced with mineral salts (bone and dentin), (5) healing materials lubricated with proteoglycans (cartilage and inverterbral disk), and (6) other special tissues that must be strong and yet have unusual properties such as the light-transmitting cornea and fatigue resistant heart valves (Eyre, 1980). It is now clear that collagen describes a family of specialized molecules that has evolved for a particular structural function outside the cell.

Structural Features of the Molecule

The distinct property of all collagen molecules is the triple helix, a unique protein conformation that is a coil of three polypeptide

subunits, or alpha chains. Each of these chains is coiled into a left-handed helix with about three amino acids per turn, and the three chains are wound together in a right-handed superhelix to form a rodlike molecule about 1.4 nm in diameter (Ramachandran and Redi, 1976). In most collagens the alpha chains contain approximately 1000 amino acid residues. Glycine is the only amino acid small enough to occupy the restricted space in which the three helical alpha chains come together in the center of the triple helix. Collagen is first synthesized as a larger molecule, procollagen. At both ends of the procollagen molecule there are terminal sequences termed "telopeptides" which are not triple helical and lack glycine at every third residue (Fessler and Fessler, 1978). These telopeptides are primary sites of cross-linking in the molecule. The molecular formula of an alpha chain of the collagen molecule is $(GLY\text{-}X\text{-}Y)_{333}$, where X and Y represent amino acids other than glycine. According to Prockop et al. (1976), proline and hydroxyproline together account for about a third of the X and Y positions, approximately a quarter of the total residues, with 4-hydroxyproline limited to the Y positions. They state that restrictions on chain conformation imposed by the ring structures of proline and hydroxyproline strengthen the triple helix and stiffen the molecule. The hydroxy group of the hydroxyproline has been shown by Berg and Prockop (1973) to be essential for stabilizing the triple helix, probably via intrachain or hydrogen bonds bridged through water molecules. These investigators found that partially hydroxylated molecules synthesized by cells in which hydroxylation was experimentally

blocked, denatured at a lower temperature than fully hydroxylated molecules. The native triple helix of the procollagen molecules is thermodynamically less stable with fewer interchain hydrogen bonds. Approximately 90-100 residues of 4-hydroxyproline per alpha chain are necessary to maintain the triple helix of collagen molecules in solution at a body temperature of 37 C (Prockop et al., 1979). The occurrence of hydroxyproline in collagen is of unique interest because in vertebrates this amino acid has been found only in a few other proteins, such as elastin (Grant and Prockop, 1972), the Clq subcomponent of the complement system (Porter and Reid, 1978), and the tail structure of acetylcholinesterase (Rosenberry and Richardson, 1977).

Genetically Distinct Collagens

There are approximately seven different collagen alpha chains that have been identified as distinct gene products in higher animals (Miller, 1976). As a broad concept, the different collagen types appear to be segregated into two major classes as judged by their location outside the cell. The most common are the fibrillar, interstitial collagens that form the bulk of the extracellular fabric of the major connective tissues, such as skin, bone, tendon, ligaments, and cartilage. Another class of less abundant collagens appears to be located in the immediate pericellular environment, probably as a part of the cell's external skeleton. The collagens found in the basal lamina of epithelial layers are considered a part of the latter; they are poorly defined and have a finer texture.

The genetically distinct collagens (Table A) are known as (a) type I, found in bone, tendon, and skin; (b) type II, especially found in cartilage and in the eye; (c) type III, associated with type I in the skin, blood vessels, and smooth muscle, and (d) types IV and V, collagen found in basement membranes. Each of these collagens is synthesized first as a procollagen molecule with distinct alpha chains (Borstein, 1974).

Biosynthesis of the Collagen Molecule

Transcription

Recently the DNA fragments corresponding to parts of the mRNA from the type I collagen has been cloned (Sobel et al., 1978). This work demonstrated that each pro alpha chain has a separate mRNA. The pro alpha chains are initially synthesized with "signal sequences" at the amino terminus (Palmiter et al., 1979). The signal sequence of the pro alpha 1 chain is different from that of the pro alpha 2 chain. The signal sequence of the pro alpha chains directs the movement of the mRNA-ribosome complex towards the endoplasmic reticulum (Blobel, 1977).

Intracellular Processing

As the nascent pro alpha chain enters the cisternae of the rough endoplasmic reticulum, the signal sequence is cleaved by a signal peptidase (Blobel, 1977). The pro alpha chains then encounter three hydroxylating enzymes located in the cisternae (Guzman et al., 1976). Two of the hydroxylases convert proline to 4-hydroxyproline or 3-hydroxyproline, and the other one converts lysyl residues to

Table A. Structurally and Genetically Distinct Collagens

Type	Tissue Distribution	Molecular Formula	Distinctive Features
I	Bone, tendon, skin, dentin, ligament, fascia, arteries, and uterus	$(\alpha 1(I))_2$ 2	Low content of hydroxylysine; glycosylation of hydroxylysine is low; broad fibrils
II	Cartilage, nucleus, pulposus, notochord, vitreous body	$(\alpha 1(II))_3$	High content of hydroxylysine; heavily glycosylated; usually thinner fibrils than type I
III	Skin, uterus, blood vessels, "reticulin fibers"	$(\alpha 1(III))_3$	High content of hydroxyproline; low content of hydroxylysine; few sites of hydroxylysine glycosylation; interchain disulfides; long carboxyl telopeptide
IV	Basement lamina, kidney glomeruli, Decemet's membrane	$(\alpha 1(IV))_3$	High content of hydroxylysine; rich in 3-hydroxyproline; retains procollagen extensions
V	Basement lamina of smooth and striated muscle; exoskeleton of fibroblast	$\alpha A(\alpha B)_2$	High content of hydroxylysine; heavily glycosylated

hydroxylysine (Cardinate and Udenfriend, 1974). The residue hydroxylated must be located in a peptide and must occupy the correct position in the amino acid sequence of the peptide; the peptide must also have a nonhelical conformation (Kivirikko, 1963). This requirement for a nonhelical conformation has a very important role in processing the collagen protein. The pro alpha peptide must have approximately 90 residues per alpha chain of 4-hydroxyproline to form a triple helix that is stable at 37 C. Once the lysine residues in the pro alpha chains become hydroxylated, sugar residues are added to the resulting hydroxylysyl residues. Two enzymes are involved in this reaction, a galactosyl transferase and a glucosyltransferase. The first enzyme adds galactose to hydroxylysine and the second enzyme adds glucose to galactosylhydroxylysyl residues (Myllyla et al., 1977). The glycosylating enzymes also require that the pro alpha chains be in a nonhelical conformation; they do not act on triple-helical substrates.

According to Oikarinen et al. (1976a), the addition of sugars to nascent polypeptide chains begins shortly after the N-terminus moves into the cisternae of the endoplasmic reticulum and hydroxylysine is synthesized. Sugars may be added after the chains are released from the ribosomes but cease when collagen folds into a triple helix.

Another important process in the synthesis of pro alpha chains is the formation of intrachain (Becker, 1976) and interchain disulfide bonds (Fesseler and Fesseler, 1978). The relationships between the formation of the interchain links in the propeptide to the processing

of the collagen domain is that the interchain disulfide bonds are among the essential requirements for folding into the triple helix (Uitto and Prockop, 1973). Their studies with cells in vitro show that the usual sequence of events is: (1) hydroxylation of the pro alpha chains, (2) synthesis of the interchain disulfide bonds, and (3) formation of the triple helix. If the hydroxylation of prolyl residues is prevented or the synthesis of interchain disulfide bonds inhibited, the molecule will not become triple helical and will remain as a nonhelical protein.

Secretion

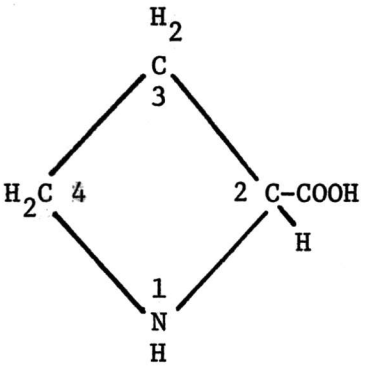
Procollagen is secreted from fibroblasts in a manner similar to most secretory proteins. The protein assembles in the endoplasmic reticulum, passes through the Golgi where post-translational modifications occur and is secreted outside the cell by way of vesicles that were pinched from the Golgi. The conversion of procollagen to collagen requires two enzymes, a procollagen aminoprotease that removes the aminopropeptides and a procollagen carboxyprotease that removes the carboxypropeptides. The secretion of the procollagen molecule illustrates how the rate of extrusion depends on the intracellular processing of the protein, specifically on folding of the collagen domain of pro alpha chains into a triple helical conformation. If post translational hydroxylations are prevented, the collagen domain cannot form a stable triple helix and this molecule is slowly secreted as a nonfunctional protein.

Collagen and L-Azetidine-2-Carboxylic Acid

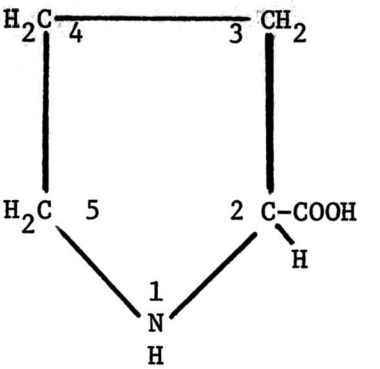
The use of several proline analogs has provided information to demonstrate that if folding of pro alpha chains into the triple-helical conformation is prevented, secretion of the protein is delayed (Uitto and Prockop, 1974b; Oikarinen *et al.*, 1976b). These investigators suggested that the incorporation of proline analogs yields pro alpha chains that are normal in size and become disulfide linked but not triple helical. Since the pro alpha chains containing analogs remain nonhelical, they are forced to react with hydroxylating and glycolating enzymes for an extended period.

The structural proline analog, L-azetidine-2-carboxylic acid (LACA), (Fig. A), has been used in several investigations of skeleton genesis during embronic development. Takeuchi and Prockop (1969) showed that when cartilage from 10-day-old chick embryos was maintained *in vitro* for 120 min in the presence of LACA, the analog replaced the proline in the procollagen synthesized by the chondrocytes. This resulted in the production of an abnormal, underhydroxylated, collegen molecule which was either retained within the chondrocyte or secreted at less than the normal rate from the chondrocyte.

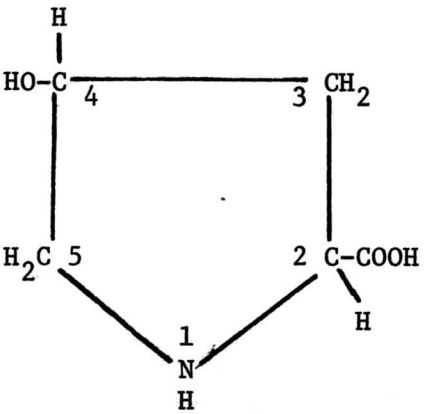
When LACA was administered to chick embryos *in ovo* between 8 and 12 days of incubation, the analog reduced both the total concentration of hydroxyproline per embryo and the extracellular accumulation of collagen, and resulted in embryos both smaller and more fragile than control uninjected embryos (lane, Dehm, and Prockop, 1971; Lane, Parkes, and Prockop, 1971; Lane, Prockop, Dehm, and Parkes, 1971). These investigators



(I)



(II)



(III)

Fig. A. LACA (I), Proline (II), 4-Hydroxyproline (III)

suggested that the accumulation of higher than normal levels of procollagen intracellularly decreased the rate of collagen synthesis by negative feedback inhibition. Any delayed extrusion of the molecule may have resulted from its underhydroxylation (Serafini-Fracassini and Smith, 1974). Lane and his coworkers suggested that the action of LACA on collagen accumulation was specific for collagen and not the result of non-specific toxicity because its effect on collagen was considerably greater than was its effect on accumulation of non-collagenous proteins.

The proline analog LACA has been used to explore several differentiating systems. Lane et al. (1971) have shown that daily injections of LACA to chick embryos between 8 and 12 days of incubation (a period during which embryonic growth and accumulation of collagen is rapid), significantly inhibited embryonic growth and collagen accumulation as assessed three to five hours after the last injection.

Scar tissue formation by fibroblasts and corneal development, both of which depend on normal formation, synthesis and extrusion of collagen for their cytodifferentiation, are specifically inhibited by proline analogs such as LACA and cis-hydroxyproline (Lane et al., 1972; Columbre and Columbre, 1972). If embryonic murine tooth rudiments are cultivated in the presence of LACA, the analog inhibits differentiation of both odontoblasts and of ameloblasts - an inhibition which can be reversed by the addition of proline or procollagen to the medium (Koch, 1974; Galbraith and Kollar, 1974). When mouse limb buds are maintained in organ culture in the presence of LACA, their

ability to elaborate extracellular matrices is delayed and their cytodifferentiation is slowed (Ayedelotte and Kochhar, 1972). Strudel (1975a,b) showed that in vivo application of LACA to periaxial tissues prevented the accumulation of extracellular material which normally forms around the notochord and the spinal cord. The applications of LACA also produced lesions in the adjacent vertebrae and caused inhibition of sclerotome differentiation into cartilage.

Other works have substantiated the specificity of the action of LACA as a proline analog, its action on collagen biosynthesis and secretion, and its use in exploring relationships between collagenogenesis and developmental processes.

CHAPTER III

MATERIALS AND METHODS

Source of Materials

Eggs of the common fowl, Gallus domesticus (White Leghorn Variety), were obtained commercially and kept in an incubator maintained at 37.8 C in a moist atmosphere. The surface of the eggs was swabbed with 70% ethanol prior to incubation and prior to injections. L-azetidine-2-carboxylic acid (LACA) was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio and Sigma Chemical Corporation, St. Louis, Missouri.

Injection Procedures

LACA, a proline analog, was dissolved in sterile 0.85% saline to give a 0.1% concentration (100 mg of LACA per 100 ml of saline). A pinhole was made in the shell and the underlying membrane with a small needle, and 0.6 ml of the LACA solution was injected into the air sac of each egg. Control embryos received an equivalent volume of 0.85% saline. The pinholes were sealed with paraffin and the eggs were placed in the incubator. The experiment involved triple injections - one prior to incubation, a second at 48 hr of incubation, and a third at 72 hr of incubation.

Skin Whole Mounts

Eight to ten-day-old embryos were removed from their shells and separated from their membranes. The embryos were then immersed in ice-cold 0.85% saline for approximately 8 min. The skin was removed

from the backs of the embryos by scoring with a razor blade and lifting off the skin with forceps. The skin was then placed on a clean dry slide, fixed in 70% ethanol and observed under phase contrast optics using a Bausch and Lomb Balplan Dual Viewing Microscope with 35 mm camera attachment.

Electron Microscopy

Transmission

Relatively large pieces of skin (1 cm in length, 1 cm in width) were removed from the chick embryos and quickly immersed in physiological saline to remove excess yolk and blood. The tissue was fixed in 4% glutaraldehyde buffered with cacodylate to pH 7.4 for approximately 4 hr at 25 C in a modification of Karnovsky's fixative (Karnovsky, 1965). It was prepared as follows: 2 g of paraformaldehyde were added to 30 ml of distilled water and heated to 70 C while stirring until the solution is cleared. After cooling to room temperature, 10 ml of 25% glutaraldehyde was added. The volume was increased to 60 ml with 0.2M sodium phosphate buffer, pH 7.6, and the final volume was made to 100 ml with distilled water. The 0.2M sodium phosphate buffer was made by mixing 100 ml of solution A ($NaH_2PO_4 \cdot IH_2O$; 27 g/l) with 900 ml of solution B (Na_2HPO_4; 28.39 g/l)(Mottet and Jensen, 1968).

The tissues fixed by both methods were washed in 0.2M cacodylate buffer from 1-2 hr and then post-fixed for 2 hr at room temperature in 4% osmium tetroxide buffered with cacodylate. After post-fixation, specimens were washed for 1-2 hr in cacodylate buffer and dehydrated in a series of ethanol at 0.4 C. The tissues were cleared with

propylene oxide and embedded in Spurr's low viscosity embedding medium. The following dehydration, infiltration and embedding schedule was used: 30, 50, 70% ethanol, 10 min each; 95 and 100% ethanol, 2 changes, 15 min for each change. Absolute ethanol was replaced by propylene oxide, 2 changes, 15 min each. The infiltration schedule included a 3:1, 1:1, and 1:3 ratio of propylene oxide plus Spurr's, one hour for each ratio concentration. The epon embedded tissue was cured for 16-18 hr at 65-68 C. Thin sections were cut at right angles to the skin surface with a glass knife using a LKB ultramicrotome and mounted on uncoated copper grids. Sections were stained for 12-15 min with uranyl acetate and subsequently stained with lead citrate for 15-20 min (Reynolds, 1963). The double stained sections were examined in a RCA EMU-4 Electron Microscope.

Scanning

Tissues for scanning electron microscopy were obtained from embryos between 6-10 days. The skin was mechanically removed from the embryo at days 9 and 10, but whole embryos were fixed at days 6-8; the skin was removed from the embryo before fixation. The tissues were prepared as follows:

(1) Fixation in 4% cacodylate buffered glutaraldehyde, 2-4 hr

(2) Washed in two changes of cold 0.2M cacodylate buffer, 1-2 hr

(3) Post-fixed in 4% cacodylate buffered osmium, 1-2 hr

(4) Washed again in two changes of cold 0.2M cacodylate buffer, 1-2 hr

(5) Dehydrated in increasing grades of ethanol, 15 min each

(6) Placed in increasing grades of amyl acetate, 10 min each; specimens remained in amyl acetate indefinitely.

After removal from 100% amyl acetate, the tissues were carried through the critical point dryer and then coated with carbon using a carbon sputter coater. The specimens were finally placed on silver coated stubs and viewed in a M-7 ISI Scanning Electron Microscope.

Hydroxyproline Analysis

To determine the amount of hydroxyproline found in skin from both controls and experimentals, the method of Neuman and Logan (1950), as modified by Grunbaum and Glick (1956) and Leach (1960), was employed. The uniquely high concentration of hydroxyproline in collagen makes the measurement of the former a good method for the accumulation or determination of the latter. For the quantitative histochemical study of collagen in various connective tissues, it was necessary to utilize a method for the determination of hydroxyproline in microgram quantities of tissue. The procedure of Neuman and Logan (1950) was found to be best suited to the micro scale; therefore, a procedure based on this method was utilized that allows for a thousand fold reduction in the quantity of hydroxyproline needed for analysis.

The skin was removed from the backs of the embryos in the same manner as mentioned earlier. The feathers were stripped from the older skin fragments. Sufficient tissue was collected to provide 1 gm wet weight in each sample. The collected tissue pieces were blotted on cold moistened filter paper to remove excess adhering water. They were

then rapidly weighed and transferred to pyrex test tubes and treated with acetone and/or petroleum ether for lipid extraction for 18-24 hr at 110 C. The tissue was then dried in an oven at 110 C for at least 12 hr. After drying, the tissue was hydrolyzed with 6N HCl in screw cap pyrex test tubes and placed in an oven for 18-24 hr at 110-120 C. The hydrolysates were neutralized, brought to appropriate volume, and filtered if necessary.

The analysis of hydroxyproline determination was performed on the neutralized hydrolysate according to Leach (1960). The standard solutions of hydroxyproline were prepared according to Leach. Absorbance of the reaction product for standards and test material was measured at 555 nm in a Bausch and Lomb Spectronic 21.

Light Histology

Skin was removed surgically from the dorsal region of the embryo and subsequently fixed in either Zenker's Fixative or 10% Neutral Formalin, washed overnight and dehydrated through a series of ethyl alcohols. The specimens were later cleared in toluene, infiltrated and embedded in carowax or paraplast, and sectioned on a rotary microtome at 10 μ. The tissues were then stained by the Mallory's Triple Connective Tissue Stain and Masson Trichrome Stain procedures (Humason, 1968).

CHAPTER IV

EXPERIMENTAL OBSERVATIONS

Macroscopical Observations

Lewis and Hunter (1978) found that a single injection of 0.9 cc of LACA to developing chick embryos between 16-48 hr of incubation, either suppressed or completely inhibited the normal development of feathers and skin. The viability of the embryos treated in this analysis was a little less than 50% of the total injected population. Of the 316 LACA-treated embryos that were viable, 64 expressed some degree of feather suppression and/or inhibition. This represented 20% of the total population.

Embryos in the present study were treated with triple injections - 0.6 cc of LACA per injection. The first injection was made at pre-incubation, the second at 24 hr of incubation and the third at 48 hr of incubation. The percent viability of these embryos did not differ significantly from that of the embryos receiving a single injection of LACA. However, the percentage of embryos with feather suppression and/or inhibition that received triple injections, did differ substantially from those receiving only single injections. The former had 35% feather suppression and the latter 20%. These observations have been summarized in Table 1.

In the previous study, embryos were injected with LACA at various ages to determine at which stage they were most susceptible to the

Table 1. Summary of data representing the total injected population, percentage of viability, and perdentage of feather suppressions following LACA administration.

	No. Embryos Injected	No. Embryos Viable	% Viability	No. *F.S.	% F.S.
CONTROLS					
0.9 cc saline	432	204	47.2	0	0
0.6 cc saline	775	362	46.0	0	0
EXPERIMENTALS					
**0.9 cc LACA	635	316	49.7	64	40
***0.6 cc LACA	1085	536	52.0	186	35

*F.S. - Feather Suppressions
**Single Injections - 0.9 cc of LACA
***Triple Injections - 0.6 cc of LACA per injection

concentration. Over a period of 0-48 hr of incubation, the number and percentage of feather suppression decreased as the number of hours increased before administering LACA (Fig. 1). However, the eggs that were injected before incubation did not develop beyond days 1 or 2; 0.9 cc of LACA at this time proved toxic to the embryo. The embryos receiving triple injections of LACA, (0.6 cc/injection), however, had an overall higher percent of feather suppression as compared to any of the single injections of LACA (0.9 cc) made at 0, 16-18, 24, or 48 hr (Fig. 1).

The LACA-treated embryos were examined grossly at various days during development because feather development is a sequential occurrence, with initial changes occurring on or around 6-8 days of development; this development continues until the 13th or 14th day of incubation. The data summarized in Table 2 and Fig 2. suggest that at certain days during development, single injections (0.9 cc) and triple injections (0.6 cc/injection) of LACA caused more visible feather suppression than at any other days. The lowest percentage of feather suppression occurred on or around days 6-8 and the highest around days 11-12. This pattern is consistent with both types of injections administered.

Light Microscopic Observations

Dorsal Skin Whole Mounts

The developing chick embryos were analyzed approximately every 12 hr (from 6-11 day) for feather pattern formation and feather growth. By the 8th day of development, the entire dorsal feather field of normal embryos is organized into a lattice system of oriented fibrils

Fig. 1. Graph represents a comparative study of the percent of feather suppressions found in LACA-treated embryos when injections were made at varying times during development.

Single injections of 0.9 cc of LACA
Triple injections of 0.6 cc of LACA;
(0.6 cc of LACA per injection)

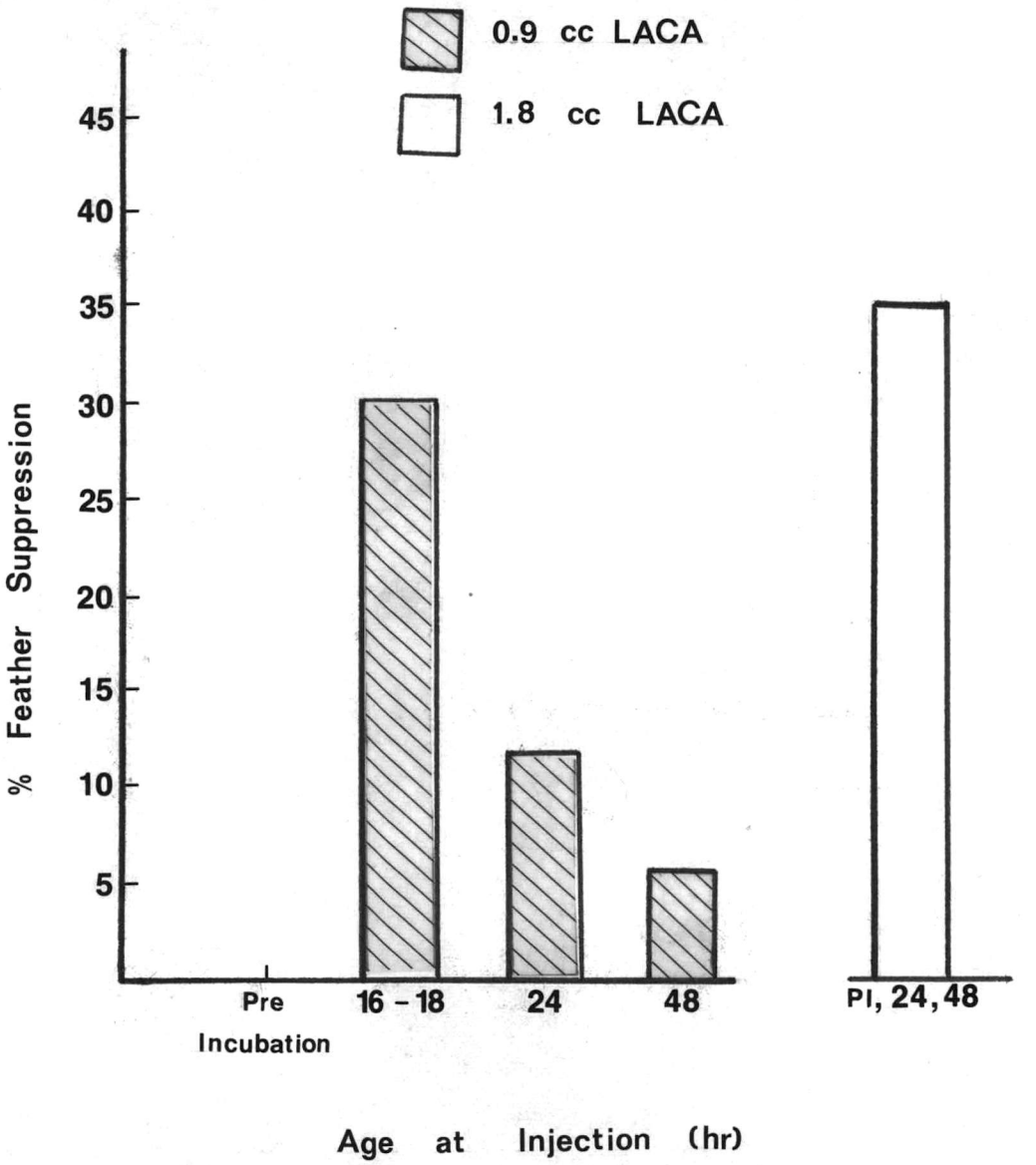

0.9 cc LACA
1.8 cc LACA
% Feather Suppression
45
40
35
30
25
20
15
10
5
Pre Incubation
16 - 18
24
48
PI, 24, 48
Age at Injection (hr)

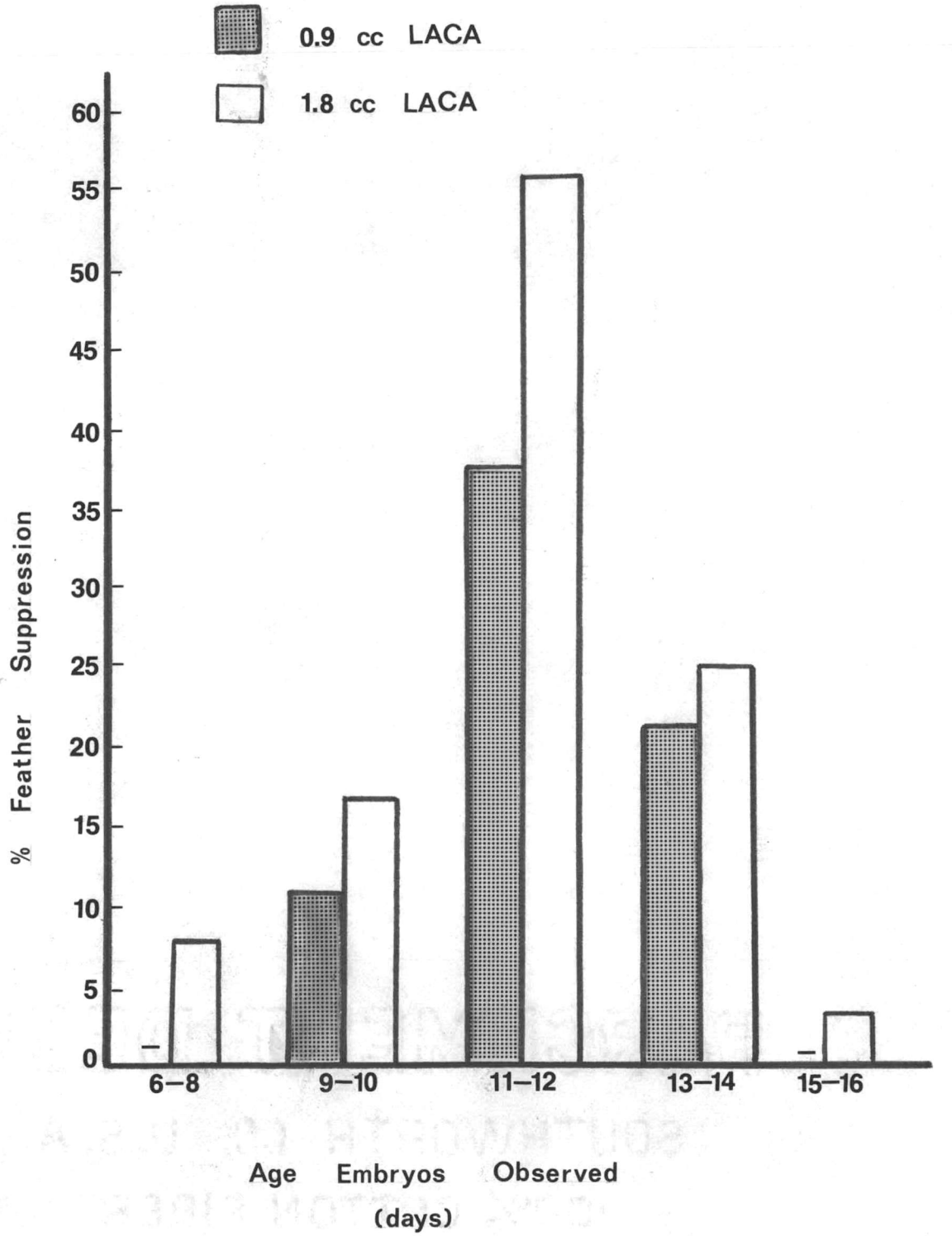
Table 2. Effects of L-azetidine-2-carboxylic acid (LACA) on feather suppression in chicken embryos at various days during development.

Groups (Age in Days)	Total No. Observed	No. of F.S.	% of F. S.
I (6-8)	71	0	0
	*112	10	8.9
II (9-10)	70	8	11.4
	106	18	16.9
III (11-12)	103	40	38.8
	101	57	56.4
IV (13-14)	72	16	22.0
	102	26	25.4
V (15-16)	-	-	-
	150	4	3.4

*Second row for each group represents data from embryos receiving triple injections of LACA; first row represents data from embryos receiving single injections of LACA.

Fig. 2. Graph represents a comparative study of the percentage of feather suppressions found in embryos treated with single injections (0.9 cc) and triple injections (0.6 cc) of LACA at various days of observation.

Single injections of 0.9 cc of LACA
Triple injections of 0.6 cc of LACA;
(0.6 cc of LACA per injection)


0.9 cc LACA
1.8 cc LACA
% Feather Suppression
60
55
50
45
40
35
30
25
20
15
10
5
0
6–8
9–10
11–12
13–14
15–16
Age Embryos Observed
(days)

linking the sites of the dermal papillae (Stuart and Moscona, 1967). Cell clusters at the lattice intersection were the precursors of the dermal papillae and arose by aggregation of cells that must migrate along the fibrous tracts of collagen. Figures 3-16 represent the dorsal skin patterns and growth of both control and LACA-treated embryos. On days 6-8 the papillae of the controls (Fig. 3) were prominent compared to those of the LACA-treated embryo (Fig. 4). Both mounts, however, did exemplify an ordered pattern of development. By the 9th day of incubation, growth has occurred in both control and experimental embryos. The growth was much more extensive in the controls. Here, too, the ordered feather pattern was apparent in the saline-treated (control) (Fig. 5) and LACA-treated embryos (Fig. 6). Although the skin mount of the 9½-day control embryo suggested that normal feather morphogenesis was occurring progressively, the skin mount of the 9½-day embryo treated with LACA showed that little if any growth has occurred within 12 hr and the distribution of papillae did not follow the normal ordered arrangement (Figs. 7-8).

In the 9½-day LACA-treated embryo (Fig. 8) where dorsal papillae should have formed, cell death seemed to have occurred. As embryonic development proceeded, the feather germs of the 10-day saline-treated embryos assumed a new distinct shape and form and the ordered array of these feathers was still obvious (Fig. 9). The ordered array of feather germs was not obvious in the 10-day skin mount of the LACA-treated embryo. Growth of the papillae was suppressed, and necrosis was seen between the developing germs (Fig. 10). Figures 11, 13, and 15,

Fig. 3. Photomicrograph of mid-dorsal skin from 8-day-old saline-treated control embryo. Normal feather pattern indicated by arrows located between developing dermal papillae (P). 10 X


P
P
3

Fig. 4. Photomicrograph of mid-dorsal skin from 8-day-old LACA-treated embryo. Normal feather pattern was not disturbed by treatment of LACA (arrows); dermal papillae (P) were suppressed by LACA treatment. 10X

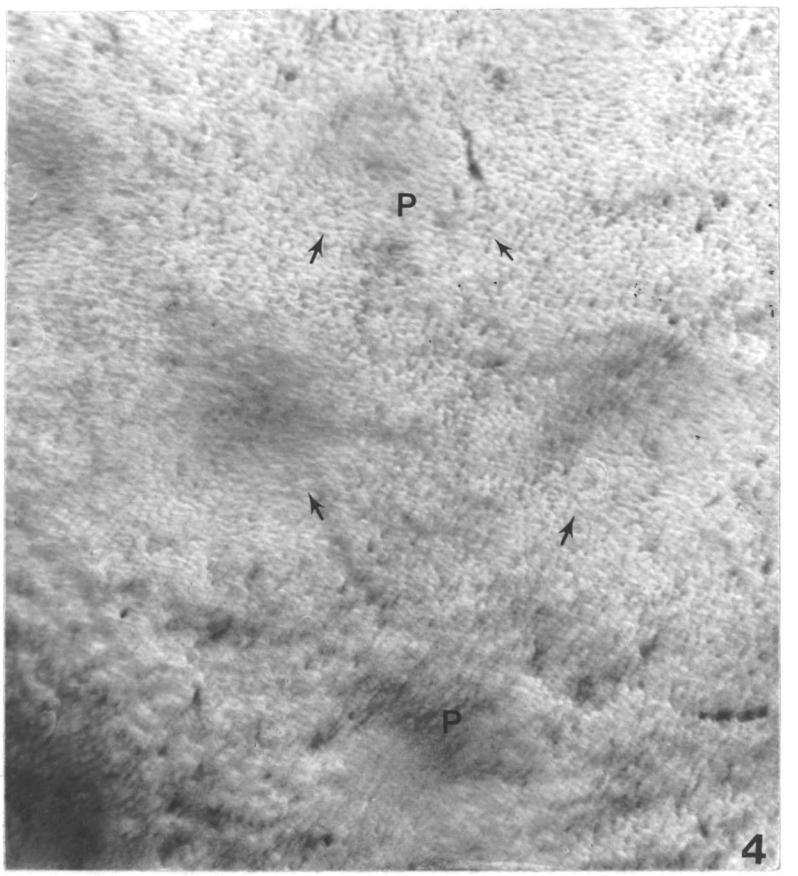

P
P
4

Fig. 5. Photomicrograph of mid-dorsal skin from 9-day-old saline-treated control embryo. The ordered pattern of feather development is evident (arrows); papillae (P). 10 X

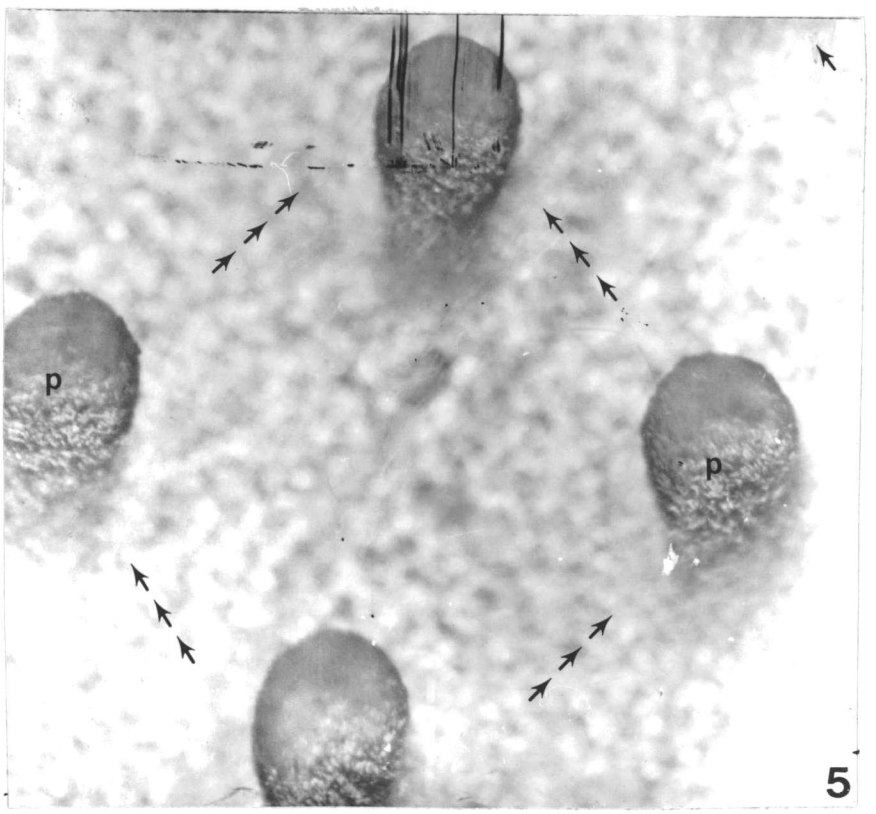

p
p
5

Fig. 6. Photomicrograph of mid-dorsal skin from 9-day-old LACA-treated embryo. The ordered feather pattern is apparent (arrow); suppressed growth of papillae (SP). 10 X

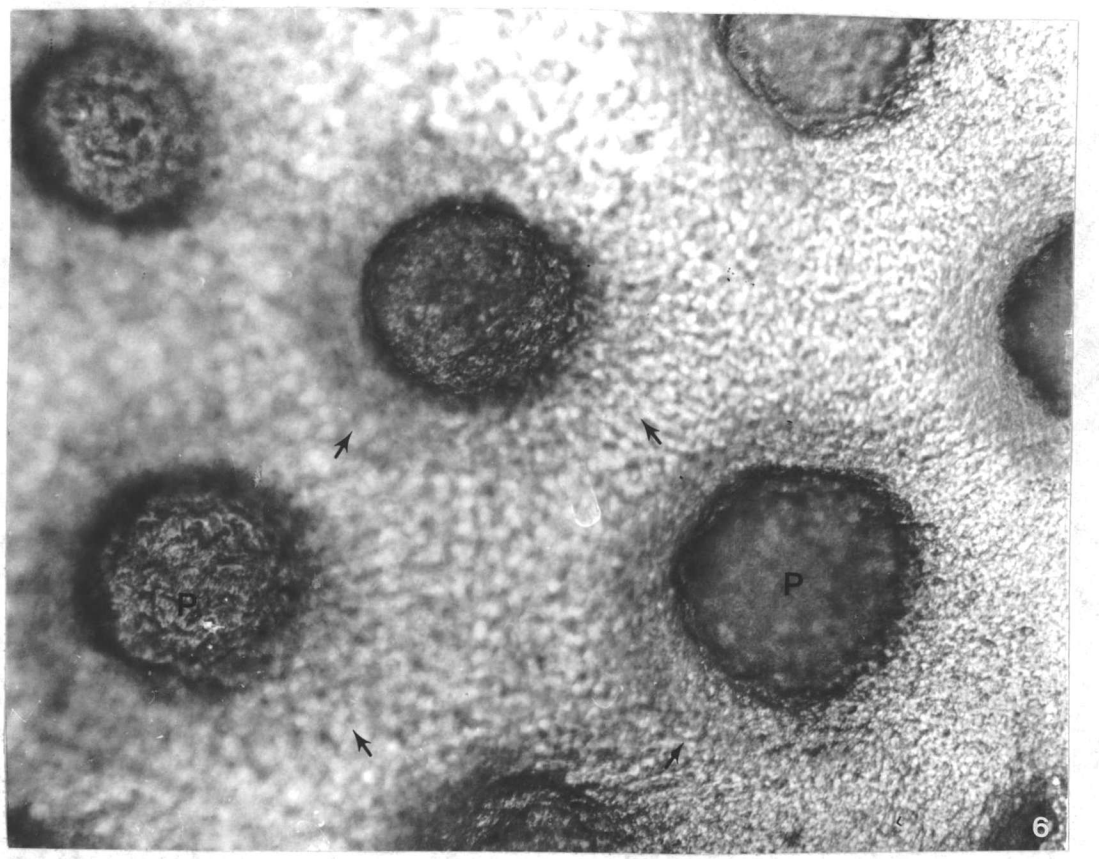

P
P
6

Fig. 7. Photomicrograph of mid-dorsal skin from 9½-day-old saline-treated control embryo. The ordered pattern of feather development is indicated by the arrows; papillae (P). 10 X


P
P
7

Fig. 8. Photomicrograph of mid-dorsal skin from 9½-day-old LACA-treated embryo. Altered feather pattern (arrows); suppressed papillae (SP); irregular epidermal surface (IES). 10 X

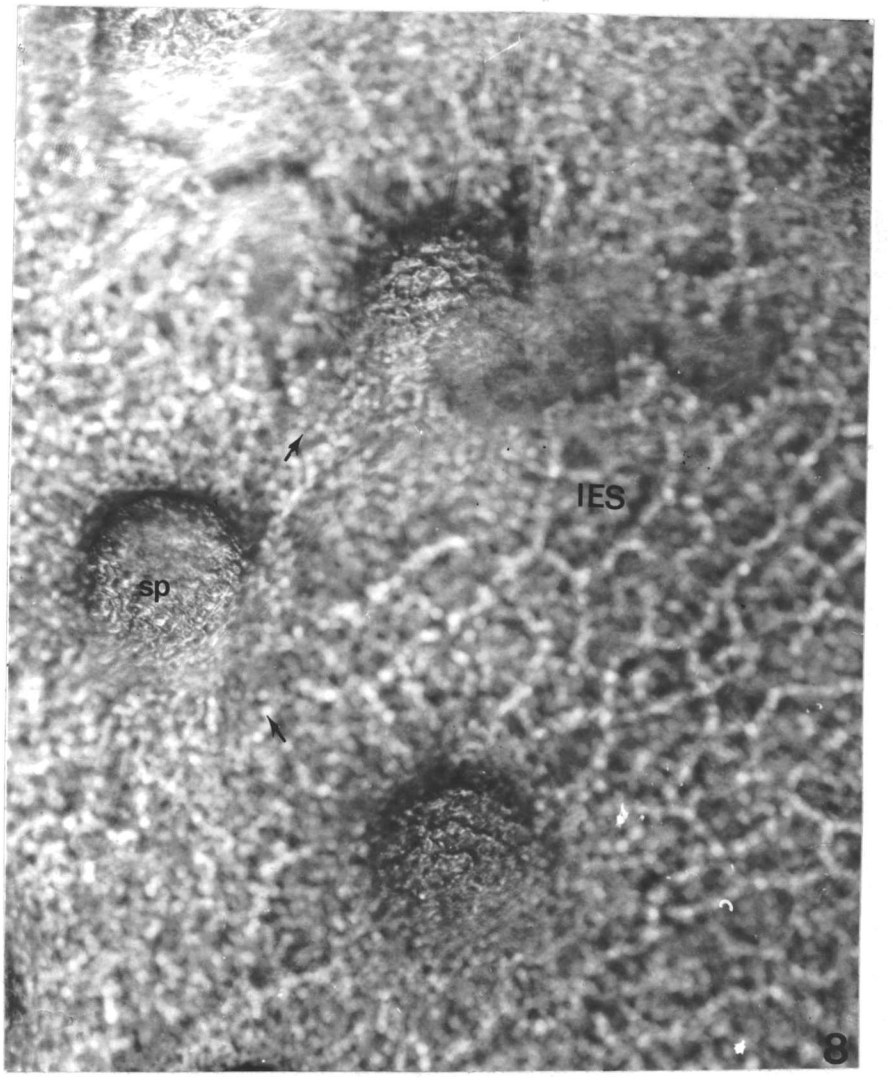

IES
sp
8

Fig. 9. Photomicrograph of mid-dorsal skin from 10-day-old saline-treated control embryo. Normal feather pattern indicated by arrows; growth and elongation of papillae (P) are apparent. 10 X


P
9

Fig. 10. Photomicrograph of mid-dorsal skin from 10-day-old LACA-treated embryo. Abnormal pattern of papillae (arrows); papillae are suppressed (SP). 10 X

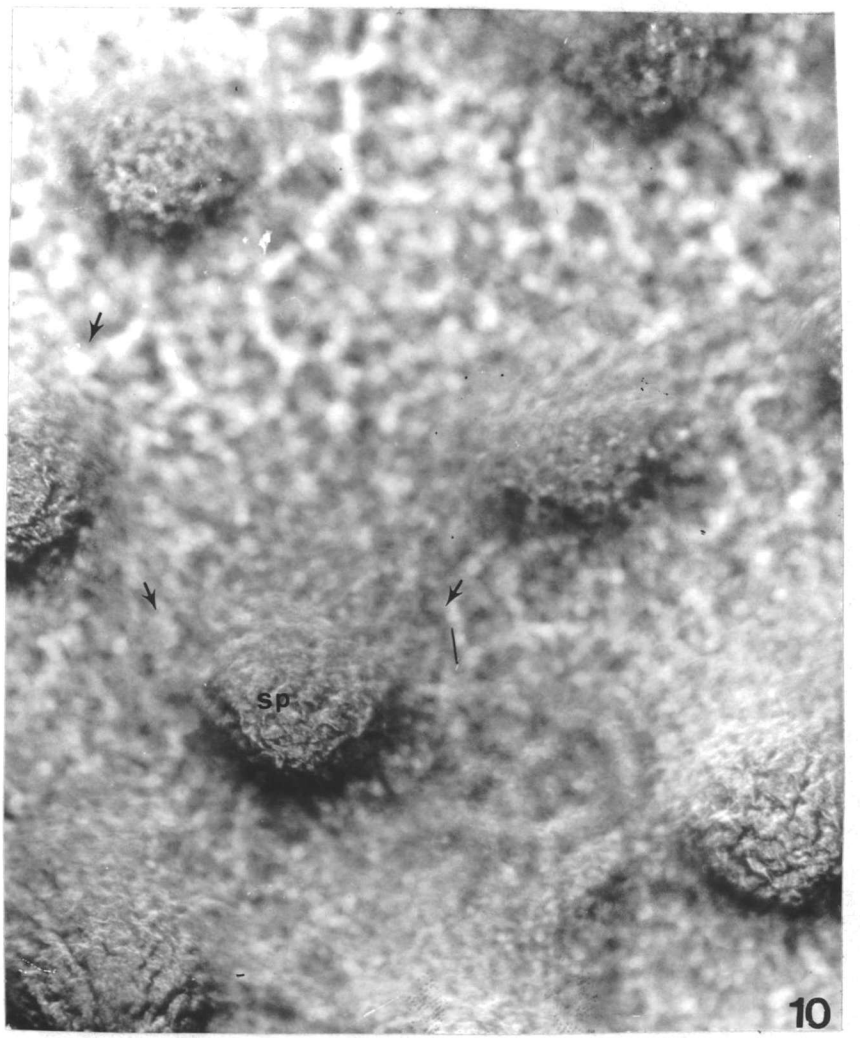

sp
10

Fig. 11. Photomicrograph of mid-dorsal skin from 10½-day-old saline-treated control embryo. Ordered feather pattern indicated by arrows; papillae (P). 10 X

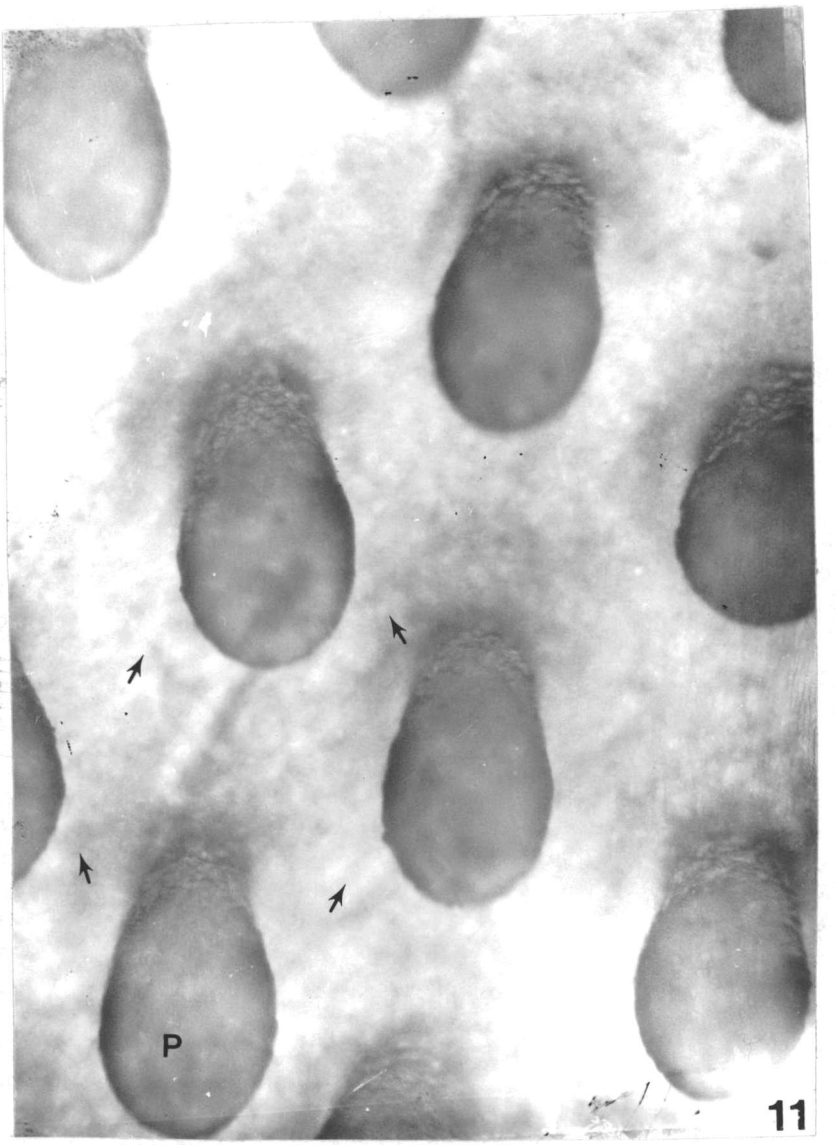

P
11

Fig. 12. Photomicrograph of mid-dorsal skin from 10½-day-old LACA-treated embryo. Papillae (P) distribution unaltered (arrows); papillae suppression (SP). 10 X

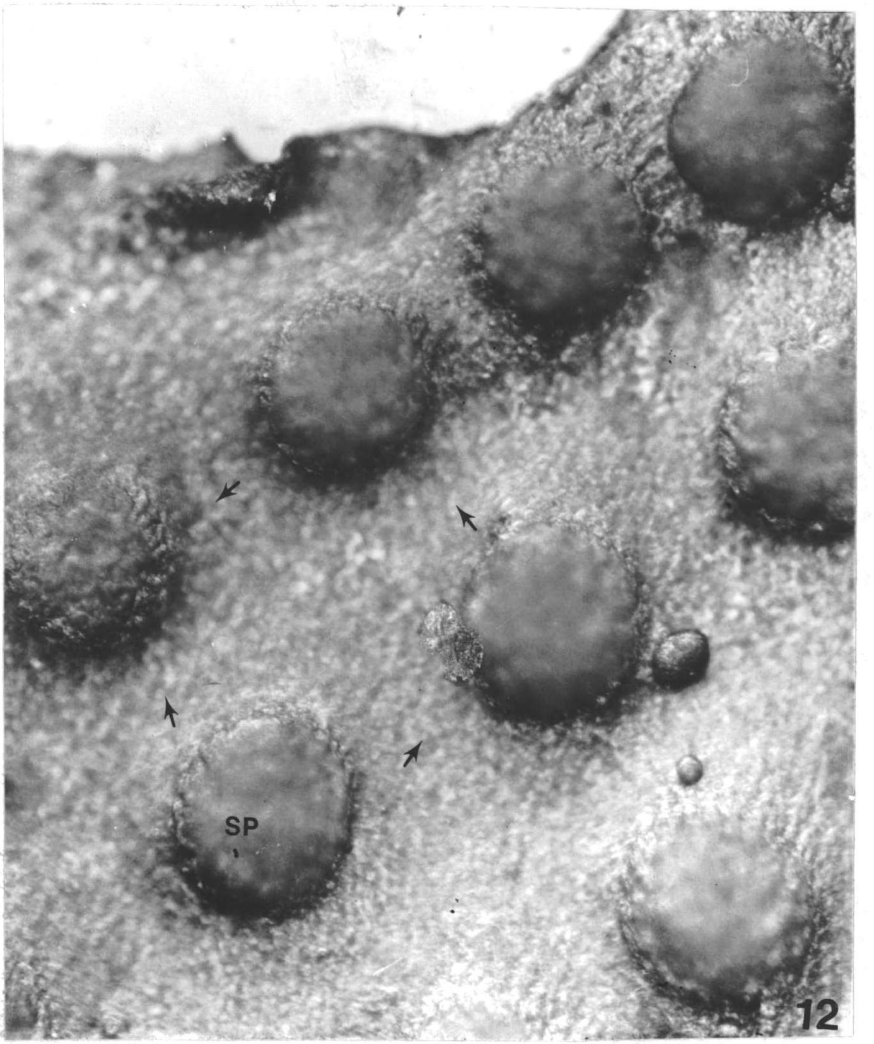

SP
12

Fig. 13. Photomicrograph of mid-dorsal skin from 11-day-old saline-treated control embryo. Normal ordered feather pattern outlined by arrows; growth and development of feathers are evident (F). 10 X


F
F
F
13

Fig. 14. Photomicrograph of mid-dorsal skin from 11-day-old LACA-treated embryo. Normal pattern of feather formation is apparent (arrows); abnormal growth of papillae (AP) outside feather tract; suppressed papillae (P). 10 X.

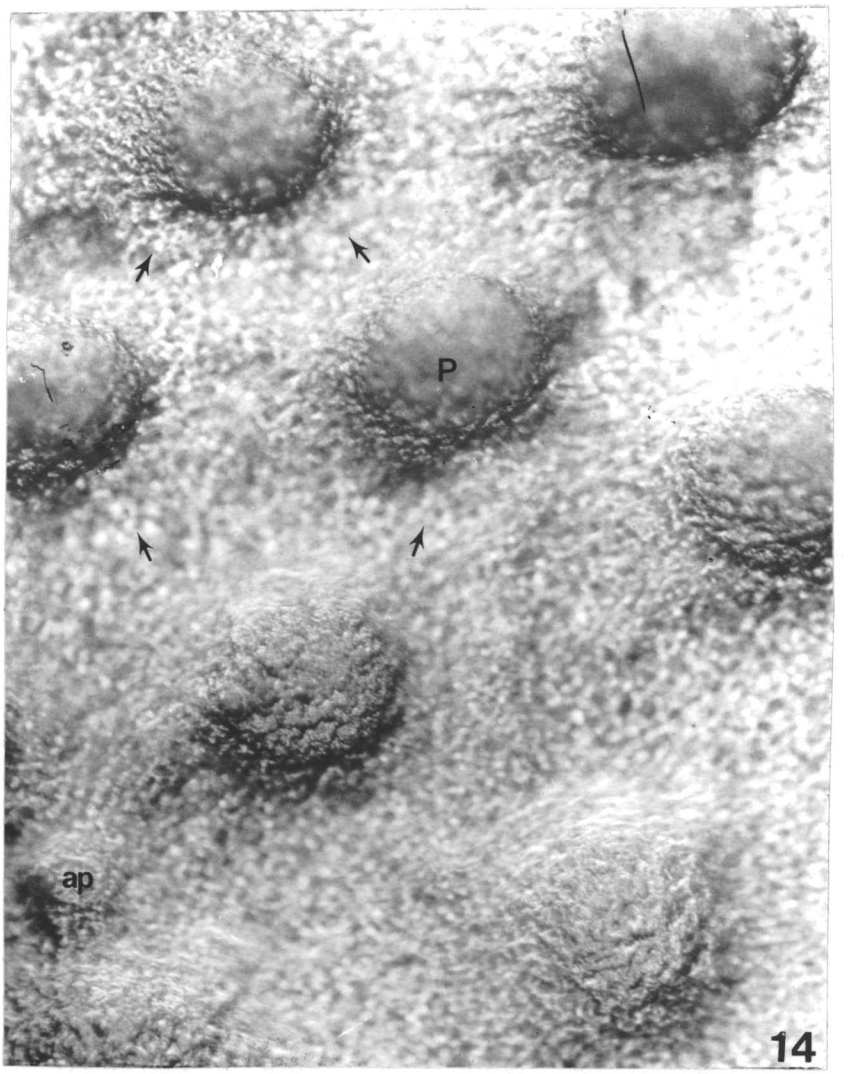
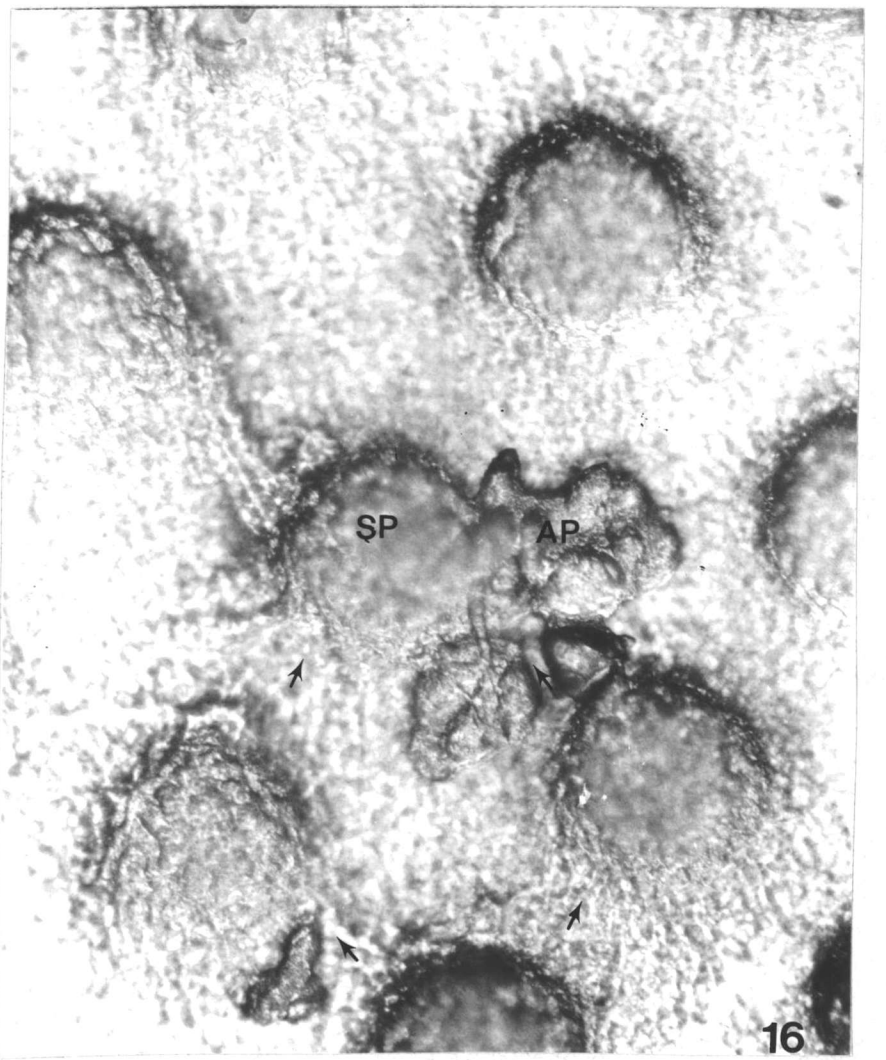

P
ap
14

Fig. 15. Photomicrograph of mid-dorsal skin from 11½-day-old saline-treated control embryo. Feather pattern indicated by arrows. 10 X


15

Fig. 16. Photomicrograph of mid-dorsal skin from 11½-day-old LACA-treated embryo. Normal feather pattern was not interrupted (arrows); papillae are suppressed (SP); growth of abnormal papillae outside the normal feather tract (AP). 10 X


SP
AP
16

day 10½, 11, and 11½ controls, respectively, show a progressive increase in growth; feather suppression is not evident. In the skin of the 11½-day-old LACA-treated embryo (Fig. 16), papillae were seen to have formed outside of the normal feather tract.

Light Histology

Dense Dermis Formation

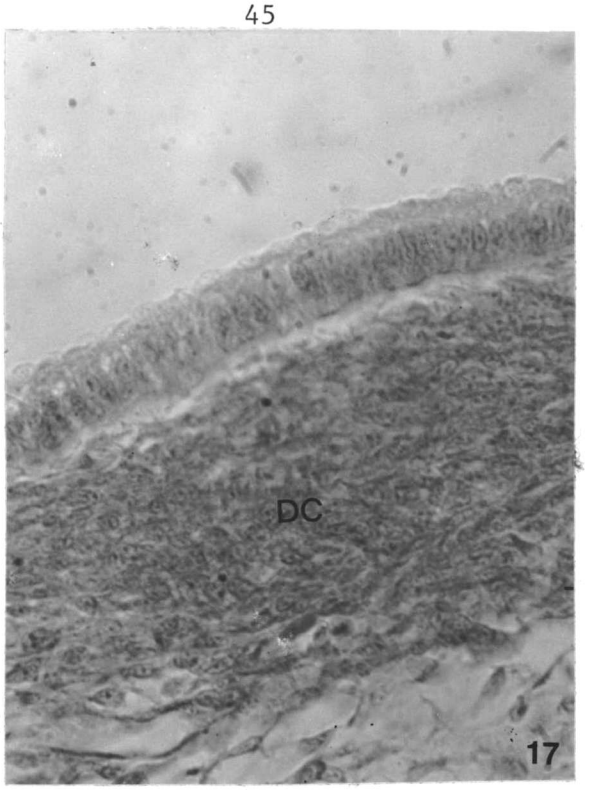
In order to assess the normal and abnormal developmental stages of the developing feather, saline-treated (control) and LACA-treated embryos were examined histologically. The first histological indication of feather development in the normal dermis (day 6) was a doubling or thickening in the density of the superficial layer of mesenchyme beneath the bilayered cuboidal epithelium (Fig. 17). In the LACA-treated embryo, the mesenchymal density apparently does not thicken as much as it does in the control (Figs. 18-19).

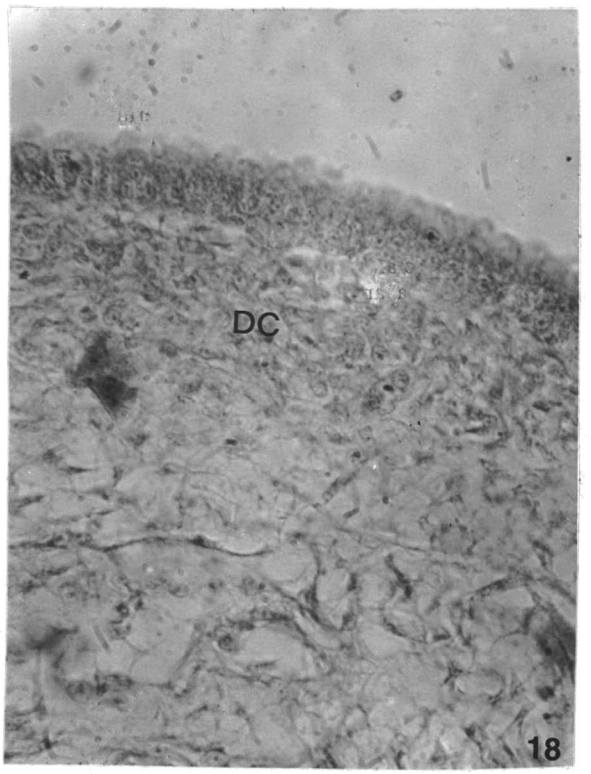
Placode Formation

The first indication of feather development in normal epidermis was the formation of a placode (day 7, Fig. 20). It appeared as a circular group of columnar epithelial cells overlaid by a single layer of flattened peridermal cells. In the LACA-treated embryo at the same age, the placode had not yet formed (Fig. 21). The discrete placode arrangement in the saline-treated embryo was replaced in the LACA-treated embryos by an epithelium comprised of a heterogeneously shaped group of columnar cells.

Fig. 17. Transverse section through mid-dorsal skin of 6-day-old saline-treated control embryo. First condensations of dermal cells (DC) covered by a uniformly thin epithelium. Mallory's Triple. 45 X

Fig. 18-19. Transverse section through mid-dorsal skin of 6-day-old LACA-treated embryo. Dermal cells (DC) are not as condensed as the controls. Mallory's Triple. 45 X


DC
17


DC
18

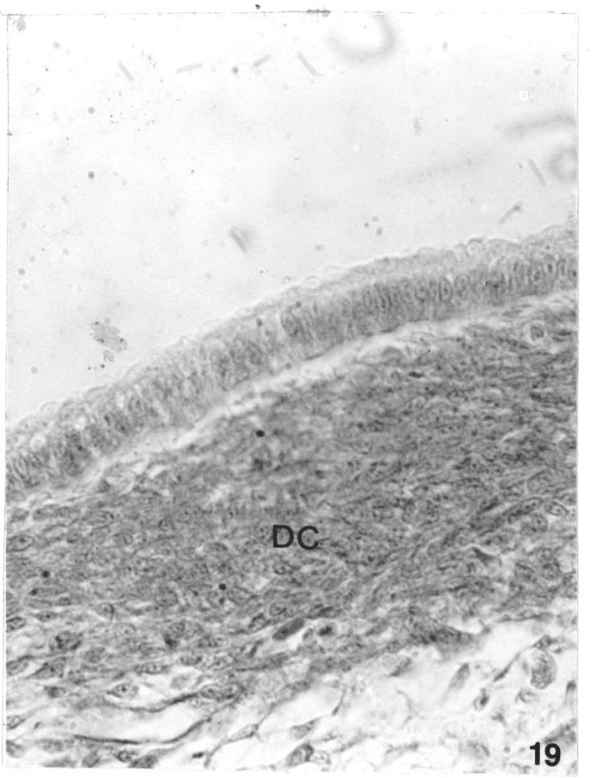
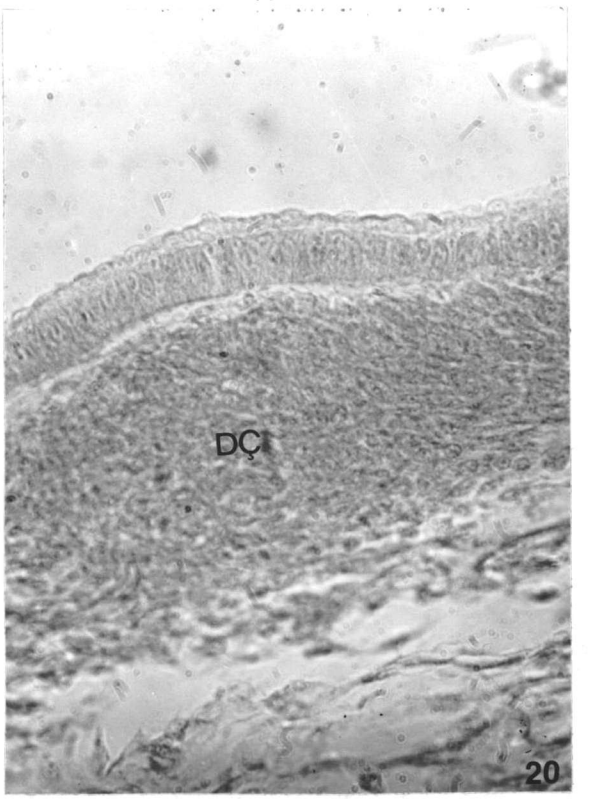
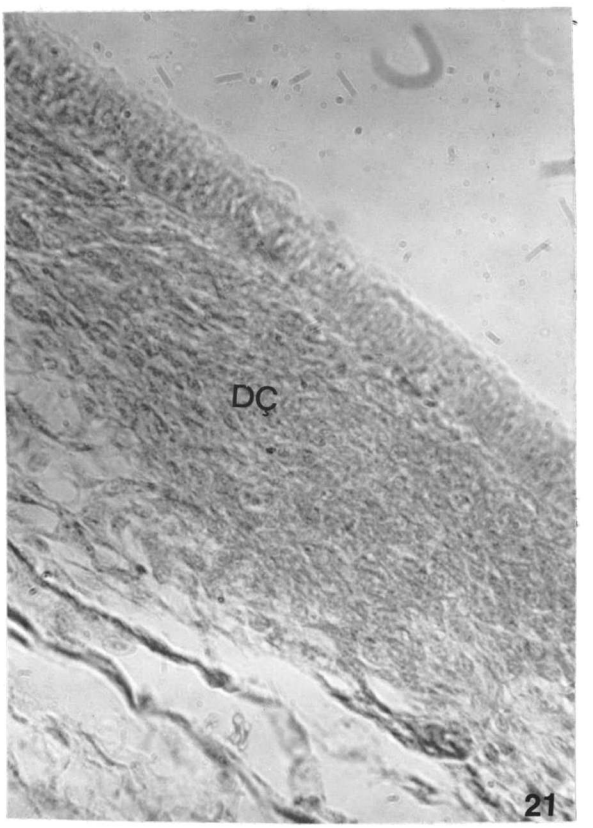

DC
19

Fig. 20. Transverse section through mid-dorsal skin of 7-day-old saline-treated control embryo. Increased dermal condensations (DC); asymmetrical epidermal placode projecting above the surface of the integument (EP). Mallory's Triple. 45 X

Fig. 21. Transverse section through mid-dorsal skin of 7-day-old LACA-treated embryo. Increased dermal condensations (DC); epidermal placode (EP) is not as thick as that of the control and does not project above the surface of the integument. Mallory's Triple. 45 X


DÇ
20


DÇ
21

Dermal Condensation Formation

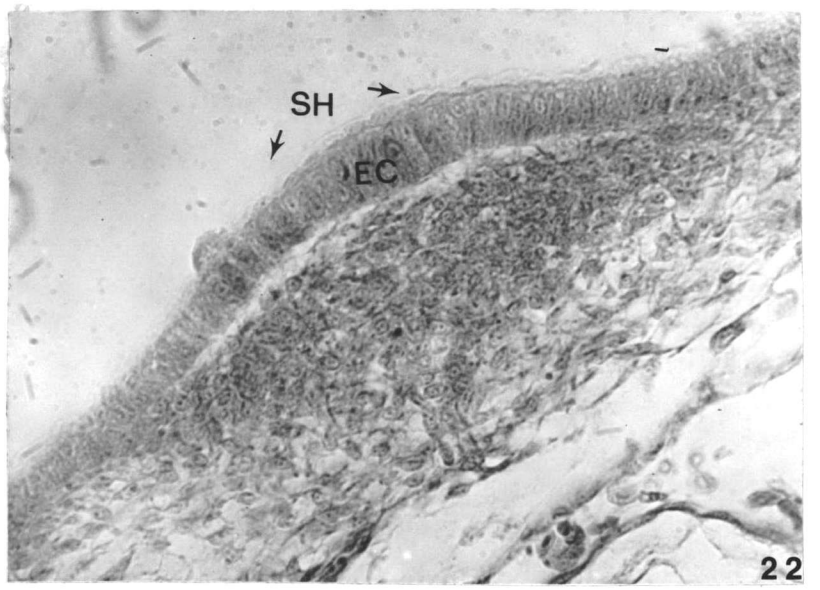
The mesenchyme condensed beneath each normal placode as a result of migration of mesenchyme and mitotic activity (days 8,9; Figs. 22,24). This mesenchymal condensation pushed or raised the ectodermal component to form a discrete feather papilla. A distinct basement membrane was completely formed by this stage in the normal embryo. The mesenchyme did not condense beneath the epidermal placode in the LACA-treated embryo; however, the dermal cells were not as compact as in the saline-treated embryo (Figs. 23,25). The basement membrane was not evident in the 8-day LACA-treated embryo but was present in the 9-day LACA-treated embryo.

Elevation of Feather Germ

During day 9 (Fig. 24), the posterior surface of the normal placode became thicker with the addition of an intermediate cell layer. By days 10-11 the apex of the feather germ was elevated above the surface of the back skin with a corresponding increase in the thickness of the underlying dermal condensations (Fig. 26-29); the feather germ had a hillock shape. Although the epithelium of the feather germ from the LACA-treated embryo acquired an intermediate cell layer, the thickness of the epithelia over the surface of these germs appeared to be unorganized with no distinct basement membrane underneath (Figs. 27,29). Thus, the polarity of the epidermis in the control feather germs, i.e., thicker epidermis on the posterior surface, was absent in the feather germs of the LACA-treated embryo. This lack of polarity in the epidermis

Fig. 22. Transverse section through mid-dorsal skin of 8-day-old saline-treated control embryo showing the initial formation of a symmetrically shaped hillock (SH), note the thicker epidermal covering of the feather germ (EC). Mallory's Triple. 45 X

Fig. 23. Transverse section through mid-dorsal skin of 8-day-old LACA-treated embryo. The initial hillock is formed but the epidermis did not thicken (E); dermal cells are not compact (DC). Mallory's Triple. 45 X


SH
EC
22

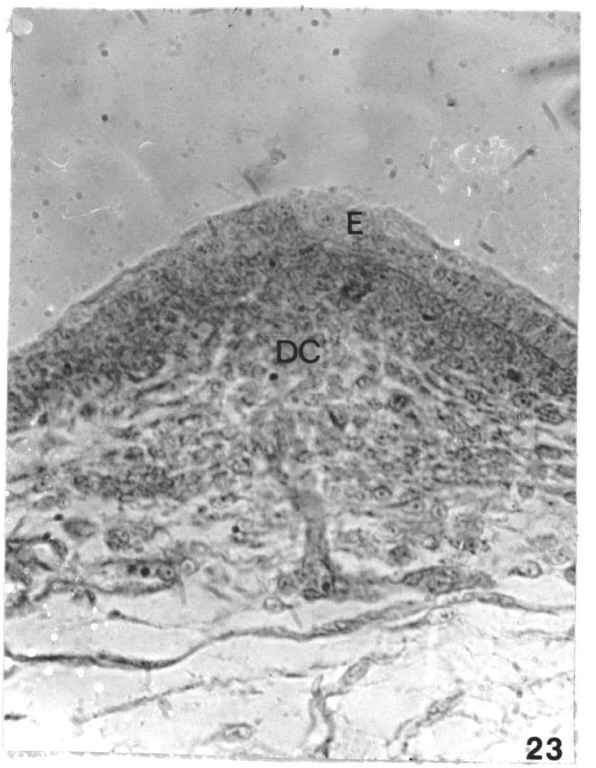
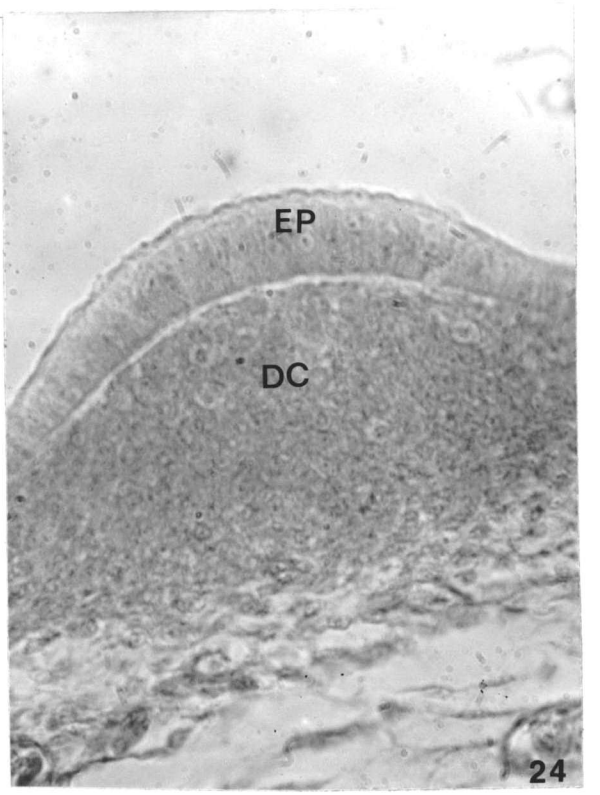

E
DC
23

Fig. 24. Transverse section through mid-dorsal skin of 9-day-old saline-treated control embryo. Epidermal placode (EP) becomes thicker with addition of intermediate epidermal cell layer; increase in thickness of dermal condensations (DC); completed hillock shape of feather germ. Mallory's Triple. 45 X

Fig. 25. Transverse section through mid-dorsal skin of 9-day-old LACA-treated embryo. Feather germ (FG) has not developed beyond initial hillock stage, dermal cells (DC) are not as condensed as they are in controls; epidermal placode does not contain intermediate cell layer (EP). Mallory's Triple. 45 X


EP
DC
24

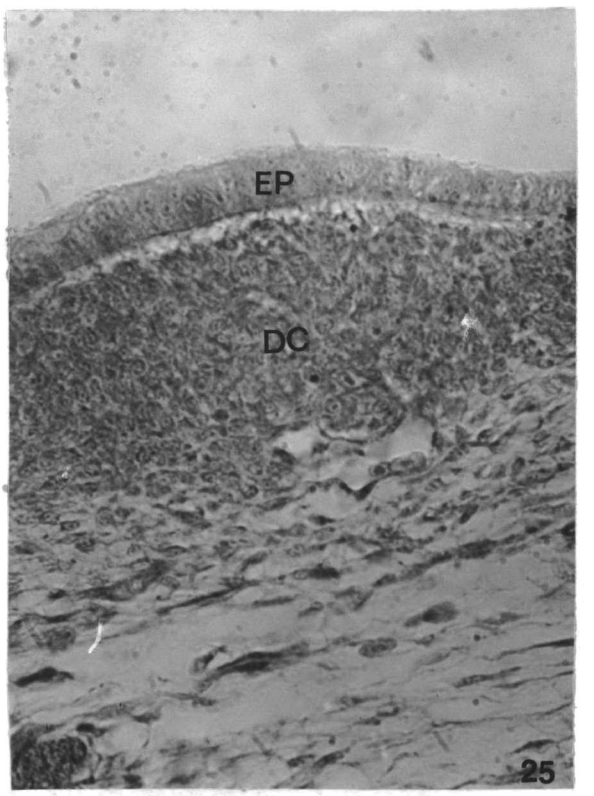
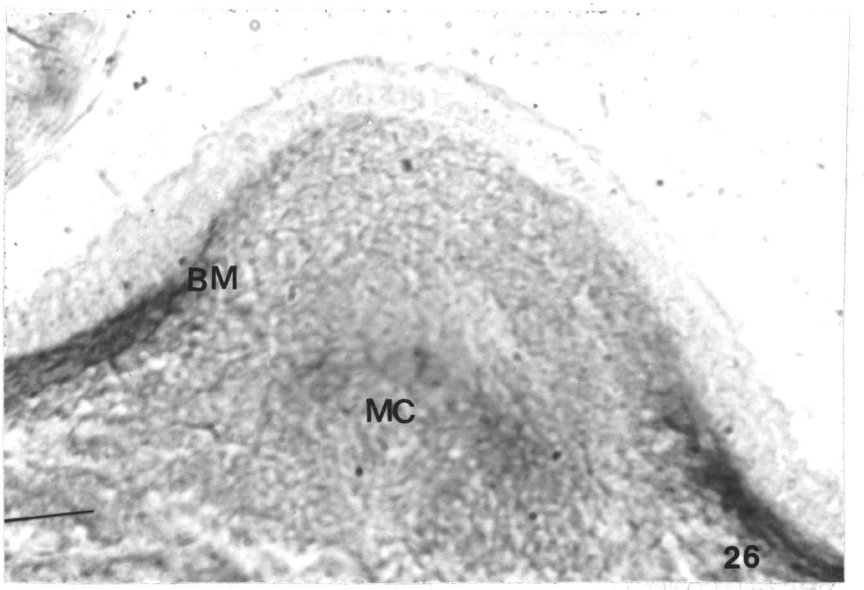

EP
DC
25

Fig. 26. Transverse section through mid-dorsal skin of 10-day-old saline-treated control embryo. Note the increased height of the dermal papilla; stained collagenous fibrils in the basement membrane (BM) and in the core of the mesodermal condensations (MC). Mallory's Triple. 45 X

Fig. 27. Transverse section through mid-dorsal region of 10-day-old LACA-treated embryo. Unorganized epidermis (E); no distinct basement membrane (BM). Mallory's Triple. 45 X


BM
MC
26

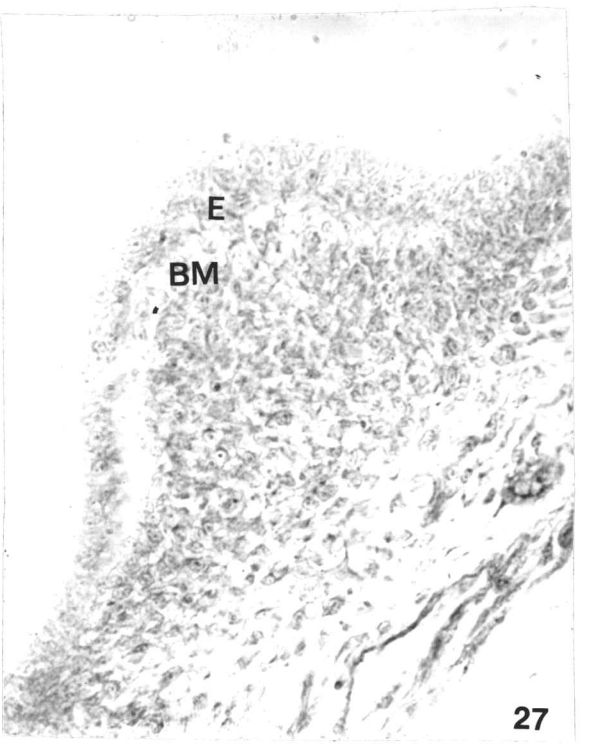
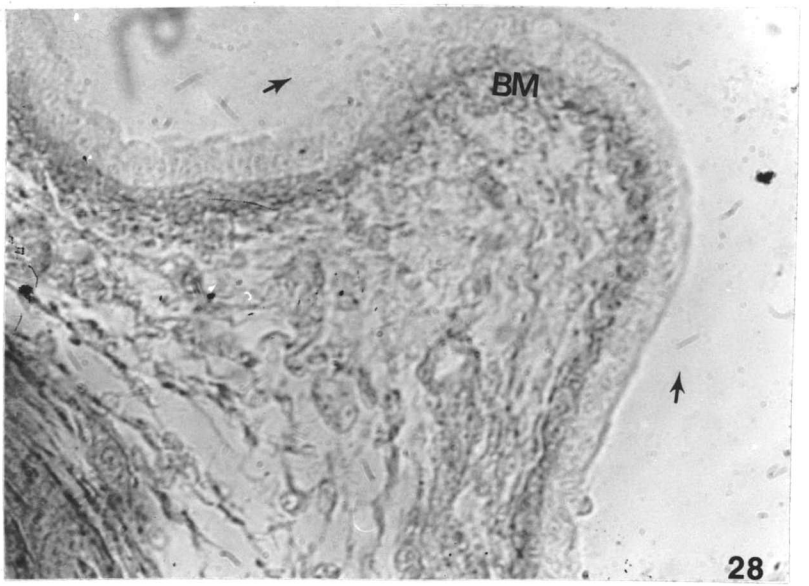
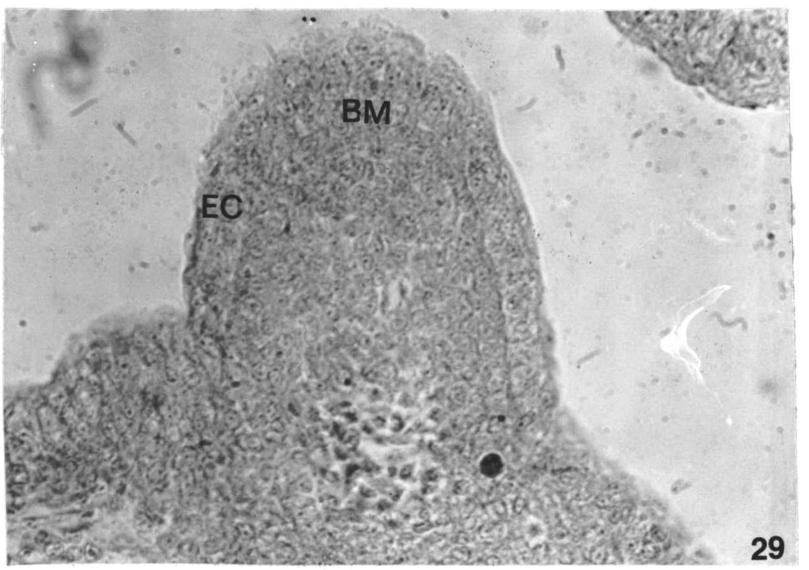

E
BM
27

Fig. 28. Transverse section through mid-dorsal region of 11-day-old saline-treated control embryo. Note increased elevation of hillock (arrows) and the distinct basement membrane (BM). Mallory's Triple. 45 X

Fig. 29. Transverse section through mid-dorsal region of 11-day-old LACA-treated embryo. Aberrant ectodermal covering (EC); no apparent basement membrane (BM). Mallory's Triple. 45 X


BM
28


BM
EC
29

was reflected in the aberrant outgrowth of the LACA-treated feather primordia (Figs. 30-34).

Elongation and Outgrowth

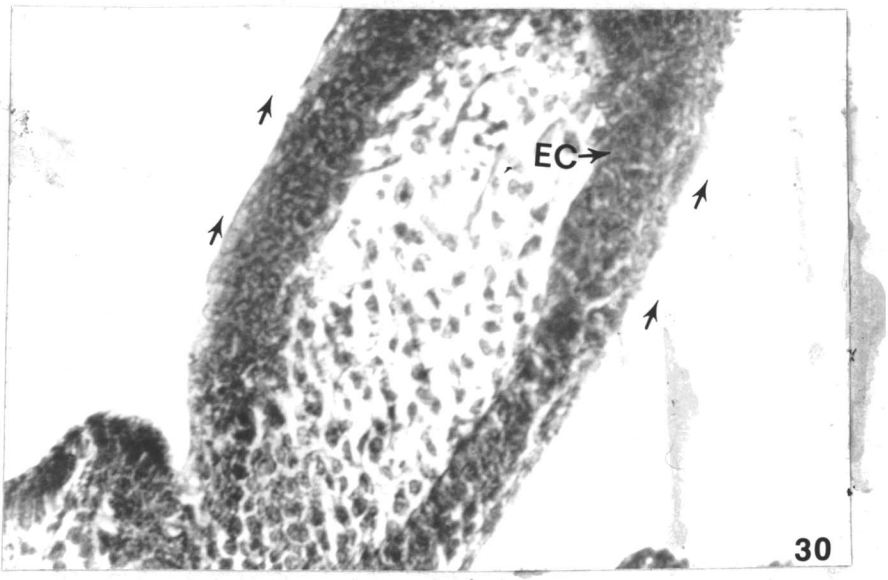
During feather morphogenesis, the thicker posterior epithelial surface of the saline-treated embryos' feather germs foreshadows the direction of their elongation and outgrowth. During day 12, the apex of the control germs projected approximately 200-400 microns above the skin's surface (Figs. 30,32). The epithelium around the apex and the posterior surface had acquired an additional intermediate layer of cells (Fig. 30). The feather germs of the 12-day LACA-treated embryo have variant shapes. The papillae in some instances did not have any distinction between the ectodermal ridge and dermal condensations (Fig. 31) and in other instances papillae revealed sparsely scattered dermal cells and an undifferentiated ectodermal ridge (day 13, Fig. 33). The ectoderm was single layered, similar to that found in 8-day-old embryos.

Barb Vane Ridge Formation

By the 12th day of incubation when the normal feather germ had elongated approximately 300-400 microns from the posterior apex to the anterior base, its epidermis had thickened greatly by the addition of more intermediate cell layers. This is the stage in which barb vane ridges were formed by the basal layers of the epidermis (days 14-16; Figs. 34-42). As the feather germs grew and elongated, more barb vane

Fig. 30. Lontigudinal section through mid-dorsal feather of 12-day-old saline-treated control embryo. Note distinct ectodermal covering (EC), and the increased elongation and outgrowth (arrow). Masson Trichrome. 45 X

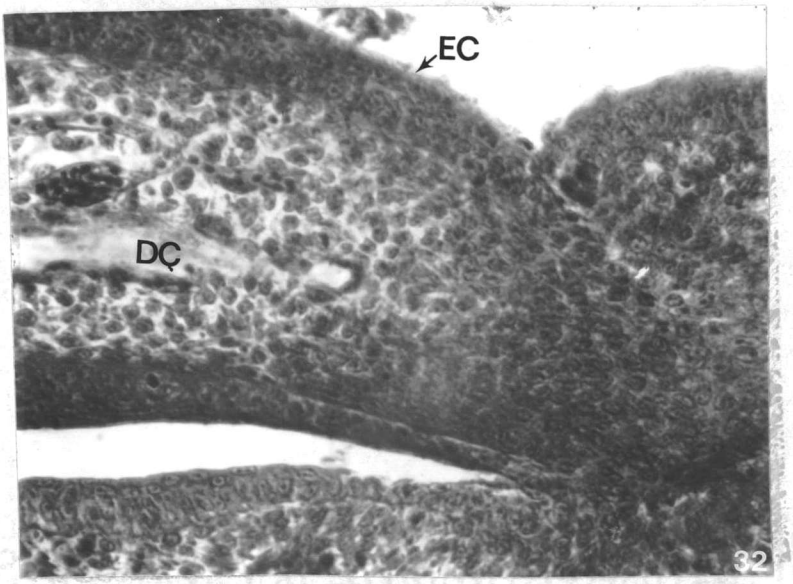
Fig. 31. Longitudinal section through mid-dorsal feather of 12-day-old LACA-treated embryo. Note lack of distinct ectodermal covering (EC), and suppressed elongation of feather. Masson Trichrome. 45 X


EC
30


EC
31

Fig. 32. Longitudinal section through mid-dorsal feather of 13-day-old saline-treated control embryo. Dermal condensations (DC), ectodermal covering (EC). Masson Trichrome. 45 X

Fig. 33. Longitudinal section through mid-dorsal feather of 13-day-old LACA-treated embryo. No apparent dermal condensations (DC); ectodermal covering (EC) did not thicken towards the apex of the feather as in the control. Masson Trichrome. 45 X


EC
DC
32

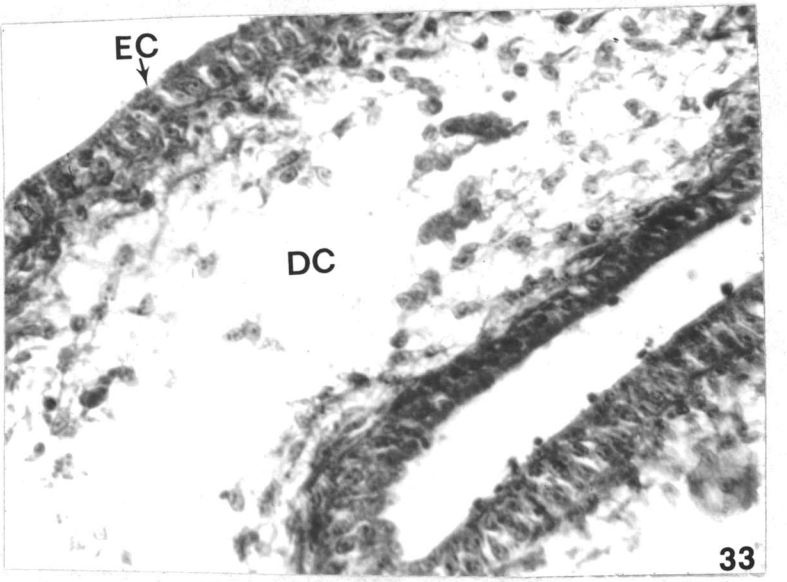
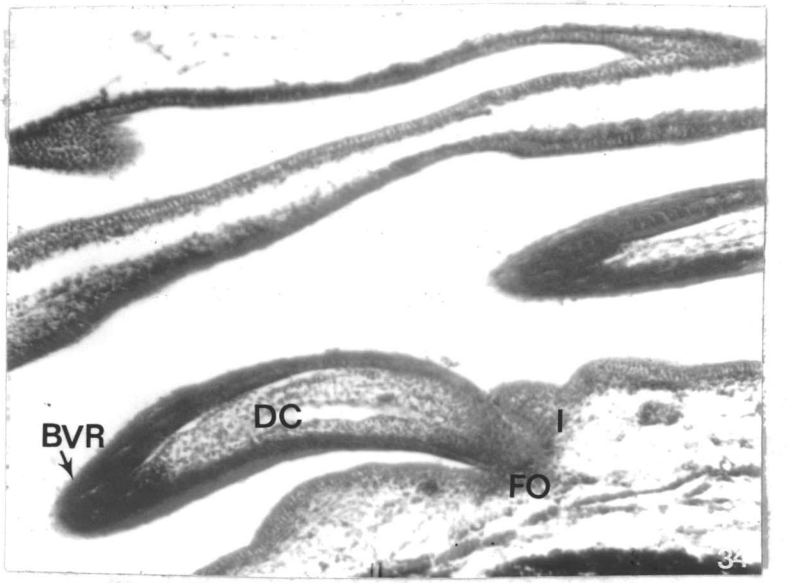
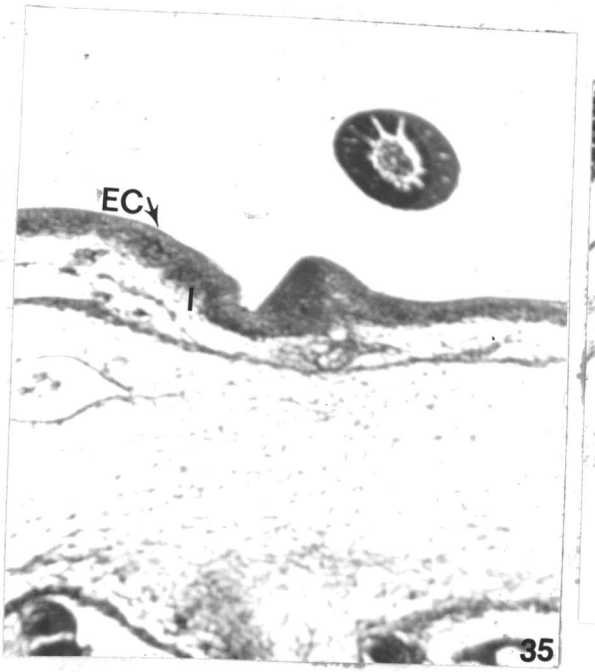

EC
DC
33

Fig. 34. Longitudinal section through mid-dorsal feather of 14-day-old saline-treated control embryo. Barb vane ridges (BVR), dermal core (DC), integument (I), feather origin (FO). Masson Trichrome. 10 X

Fig. 35-36. Longitudinal section through mid-dorsal skin of 14-day-old LACA-treated embryo. Note suppressed elongation of feathers and lack of distinct ectodermal covering (EC), integument (I). Masson Trichrome. 10 X


BVR
DC
I
FO
34


EC
I
35

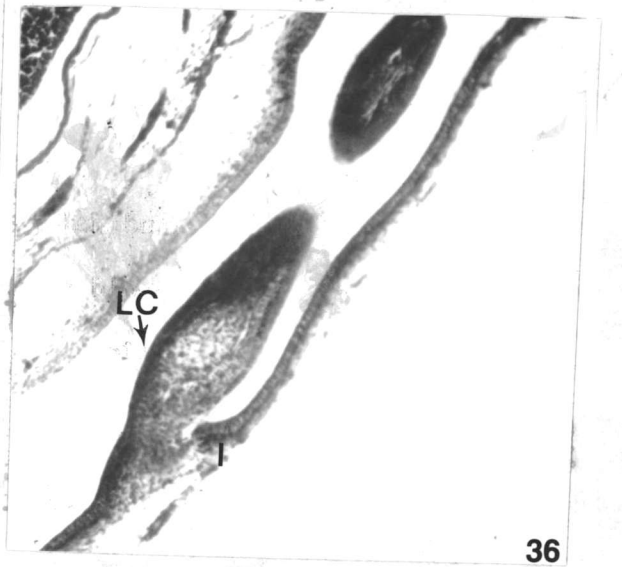
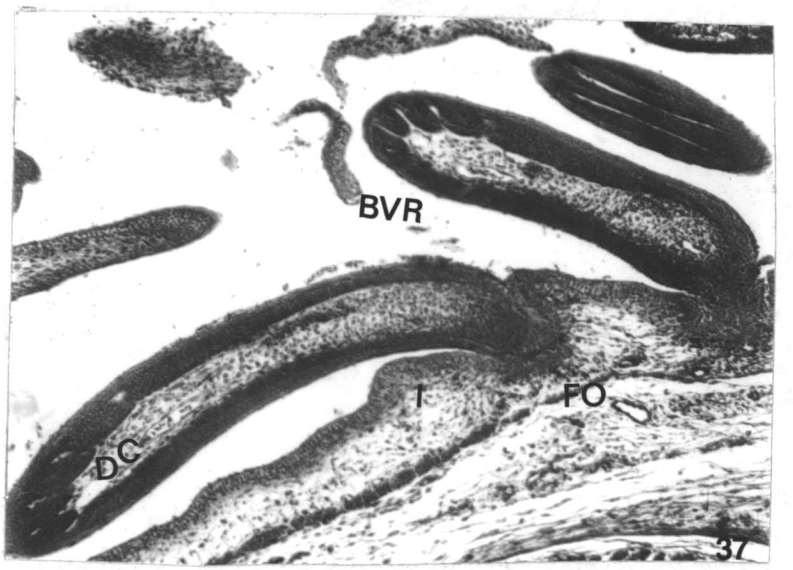

LC
I
36

Fig. 37. Longitudinal section through mid-dorsal region of feather from 15-day-old saline-treated control embryo. Barb vane ridges (BVR), dermal core (DC), feather origin (FO) and integument (I). Masson Trichrome. 10 X

Fig. 38. Longitudinal section through mid-dorsal feather of 15-day-old LACA-treated embryo. Barb vane ridges (BVR), dermal condensations (DC), feather origin (FO), integument (I). Masson Trichrome. 10 X


BVR
I
FO
DC
37

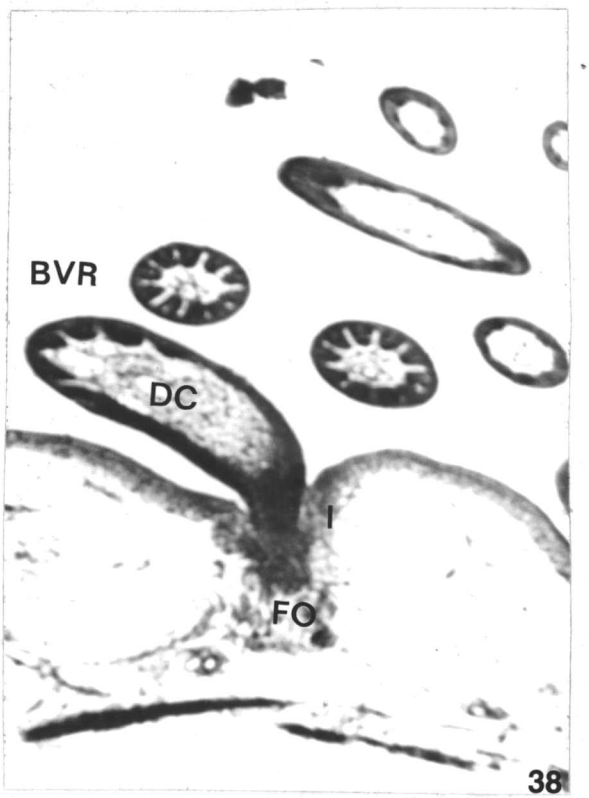
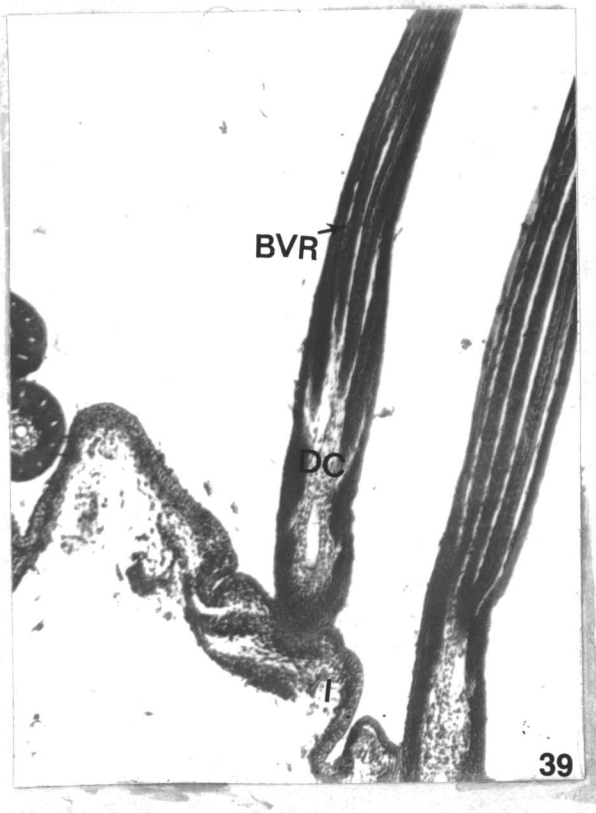

BVR
DC
I
FO
38

Fig. 39. Longitudinal section through mid-dorsal feather of 16-day-old saline-treated control embryo. Differentiated barb vane ridges (BVR), dermal core (DC), feather origin (FO), integument (I). Masson Trichrome. 10 X

Fig. 40. Longitudinal section through mid-dorsal feather of 16-day-old LACA-treated embryo. Note suppressed development of feathers (FS), lack of dermal condensations (DC), and single layered ectodermal covering (EC). Masson Trichrome. 10 X


BVR
DC
I
39

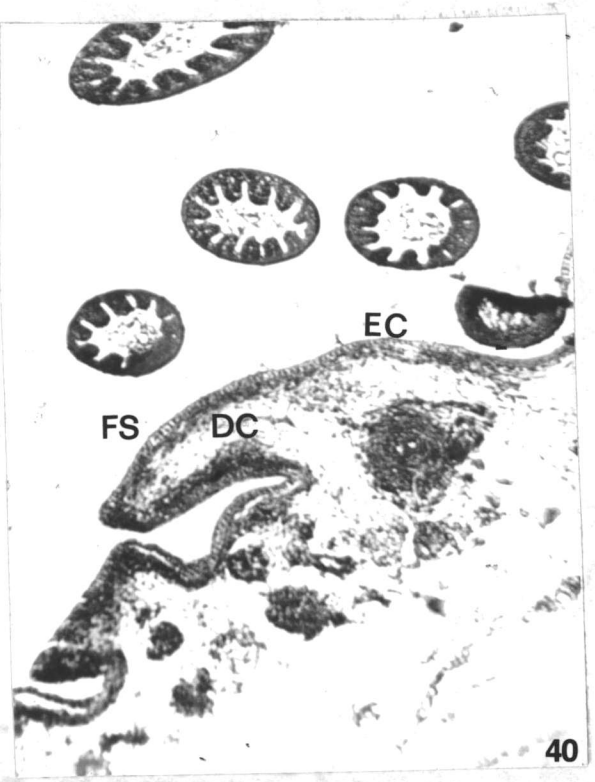
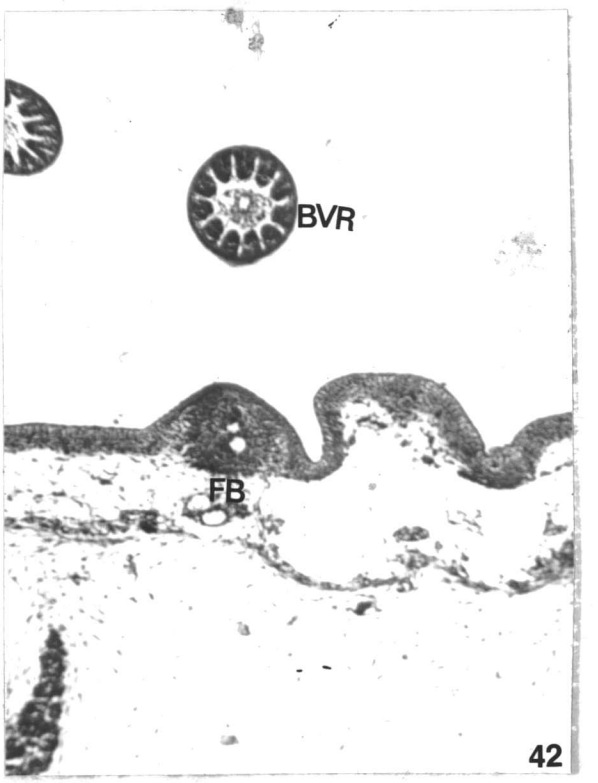

EC
FS
DC
40

Fig. 41. Transverse section through mid-dorsal skin of 16-day-old saline-treated control embryo. (FB) indicates a cross section of a feather through its base. The barb vane ridges (BVR) are seen in the cross section of the feather near its apex. Masson Trichrome. 10 X

Fig. 42. Transverse section thorugh mid-dorsal skin of 16-day-old LACA-treated embryo. Cross section of feather base (FB); the number of developing feathers within a given area of the skin is less when compared to the control; barb vane ridge (BVR). Masson Trichrome. 10 X


BVR
FB
41


BVR
FB
42

ridges appeared around the dermal core as the basal layers of the epidermis formed columns of cells (days 15-16; Figs. 37,39). The resultant configuration through a cross section of a single feather was a circular arrangement of 11-15 barb vane ridges around a dermal core (Figs. 35,38). Each barb vane ridge represented a column of cells which was a prospective barb with two rows of attached barbules and will keratinize.

In papillae of the LACA-treated embryo, the dermis in the apical region of the germs appeared less organized (Figs. 36,40). The germs' elongation process has been somewhat suppressed. However, barb vane ridges did form and were arranged in a circular configuration just like the controls (Figs. 35,36,40). In many histological sections through the dorsal skin, the distance between one developing feather and another in the experimentals, was much greater than the distance observed between adjacent feathers of the control (Figs. 35,38,42).

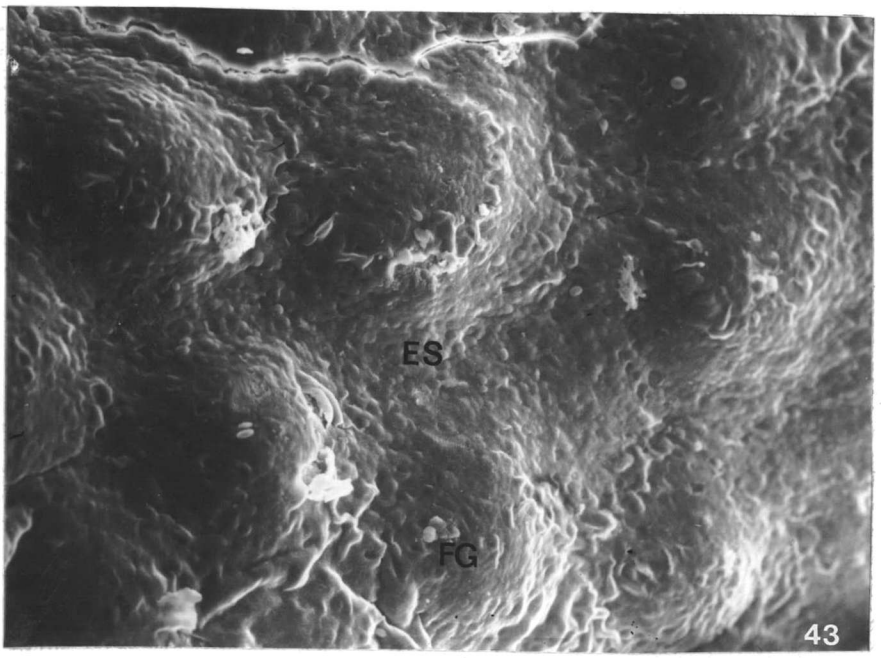
Electron Microscopic Observations

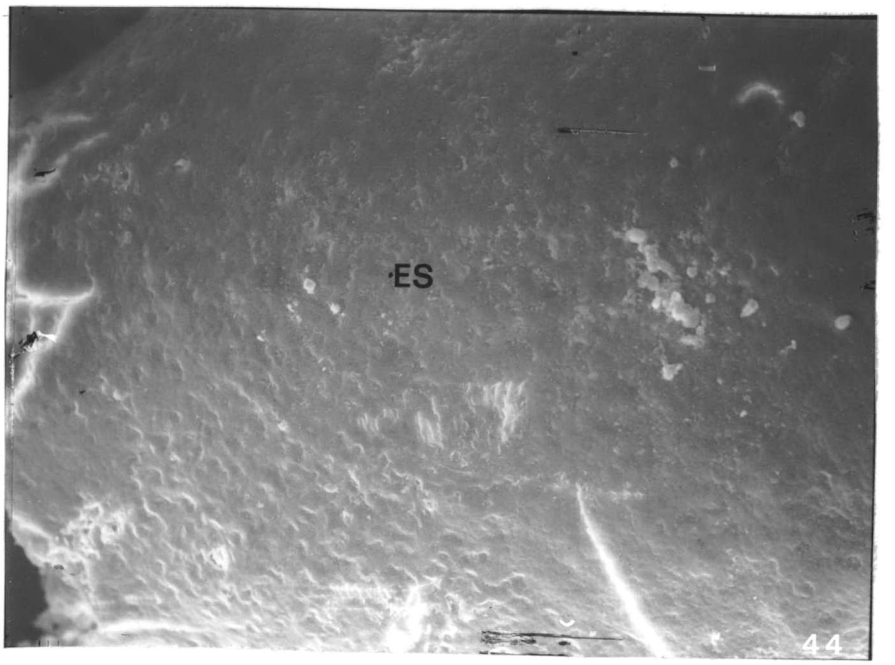
Scanning

Feather germs were seen to have already started their development in the 6½-day-old embryo's integument (Fig. 43) . However, the epidermal surface seemed to be rather fragile in nature. The skin of the 6½-day-old LACA-treated embryo, on the other hand did not show any development of feather germs (Fig. 44). The texture of the epidermal surface was similar, but the induction of the feather germs in the skin of the LACA-treated embryo was inhibited. A cross-section through the dermis of the 6½-day-old control showed the distinct collagen fibrils that are

Fig. 43. Scanning electron micrograph of dorsal skin from 6½-day-old saline-treated control embryo. Note the development of the early feather germs (papillae) (FG); epidermal surface (ES). X 217

Fig. 44. Scanning electron micrograph of dorsal skin from 6½-day-old LACA-treated embryo. Note the inhibition of the feather germs. The texture of the epidermal surface (ES) appears to be similar to that of the control. X 217


ES
FG
43


ES
44

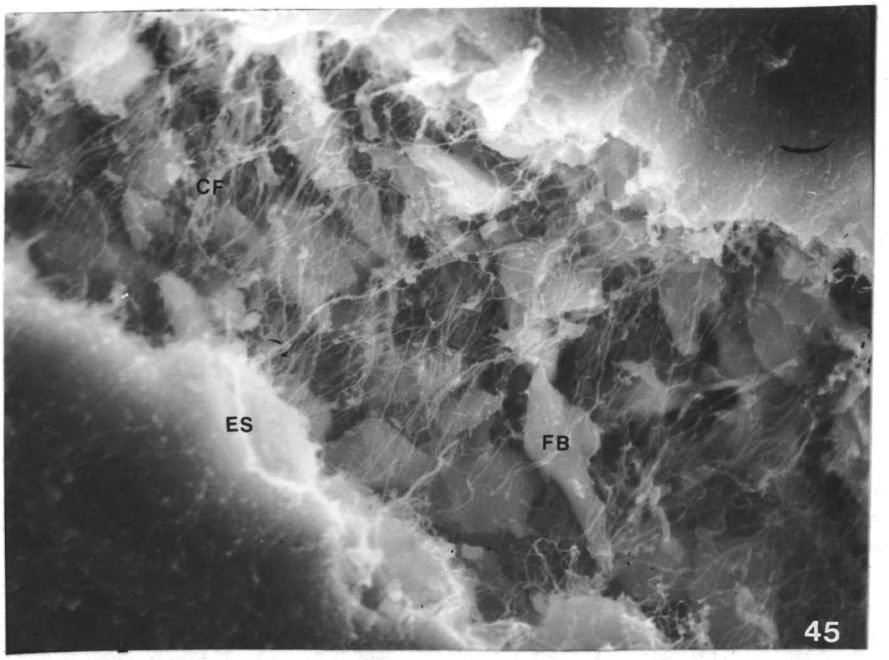
found to be interwoven among the dermal fibroblasts (Figs. 45-46). These fibers were rather abundant and closely associated with one another (Fig. 46). In the 6½-day-old LACA-treated embryo, these fibers were not apparent, and the dermal fibroblasts were not distinct (Figs. 47-48). Several other kinds of irregularities were noticeable in the 6½-day-old skin of LACA-treated embryos as shown in Figs. 49-50. Lacerations are evident in the epidermal sheath of Fig. 49 and foldings of the epidermal covering are apparent in Fig. 50.

Figures 51 and 52 are scanning electron micrographs of the dorsal skin taken from an 8-day-old control and experimental embryo, respectively. The former micrograph was taken at a lower magnification than the latter, but the dermal papillae were much more apparent in the control micrograph than in the experimental. The papillae were in the characteristic hump stage. The evaginations in the experimental (Fig. 52) have not reached the characteristic hump stage; actually the papillae have not yet formed. The numerous small protuberances seen in the micrograph represented the peridermal cells of the epidermis and not small papillae.

The papillae of the 9-day-old control have increased in size and have taken on a distinctive and characteristic shape (Fig. 53). The 9-day experimental did not show papillae with this characteristic shape. The development of the oncoming papillae in the skin of the LACA-treated embryo had only reached the stage where slightly flattened mounds protruded above the skin's surface (Fig. 54). The development of papillae in the skin of both the 8 and 9-day-old LACA-treated embryo

Fig. 45. Scanning electron micrograph: cross sectional view of the dermis of a 6½-day-old saline-treated control. Collagen fibrils (CF), dermal fibroblast (FB), epidermal surface (ES). X 2,150

Fig. 46. Scanning electron micrograph: cross section of 6½-day-old saline-treated control. An increased magnification of the above micrograph. Collagen fibrils (CF), dermal fibroblasts (FB). X 6,280


CF
ES
FB
45

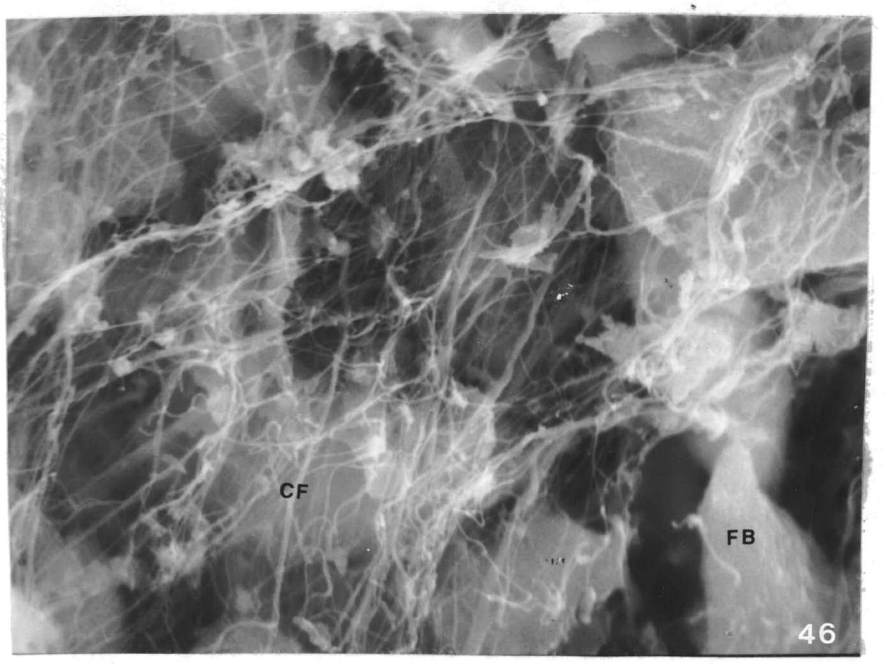
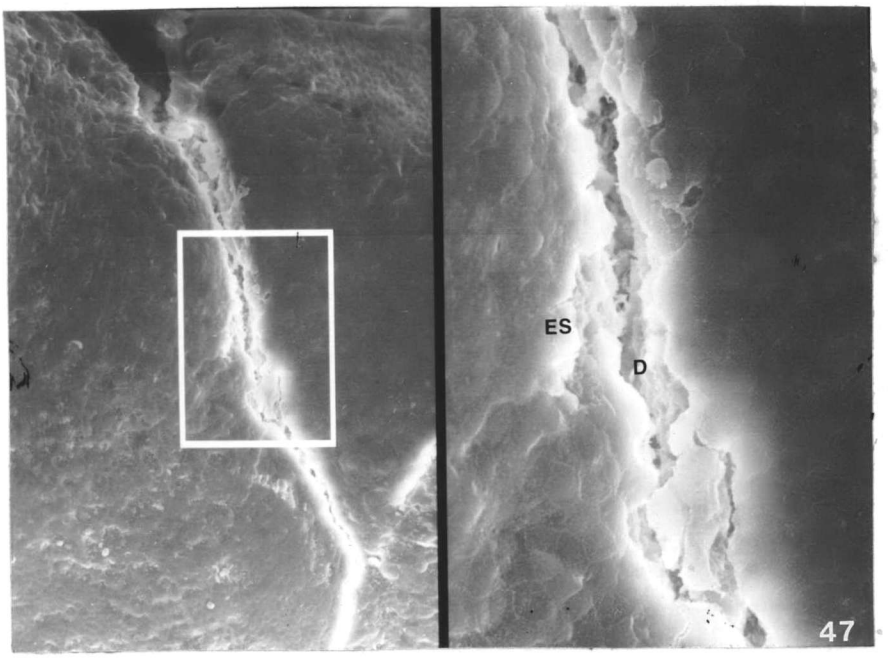

CF
FB
46

Fig. 47. Scanning electron micrograph of epidermal surface of 6½-day-old LACA-treated embryo. This micrograph provides a reference point for Fig. 48. Epidermal surface (ES), dermis (D). X 217

Fig. 48. Scanning electron micrograph: cross sectional view of the dermis of a 6½-day-old LACA-treated embryo. Note the absence of dermal fibrils and the irregular arrangement of dermal cells (DC). X 2,150


ES
D
47

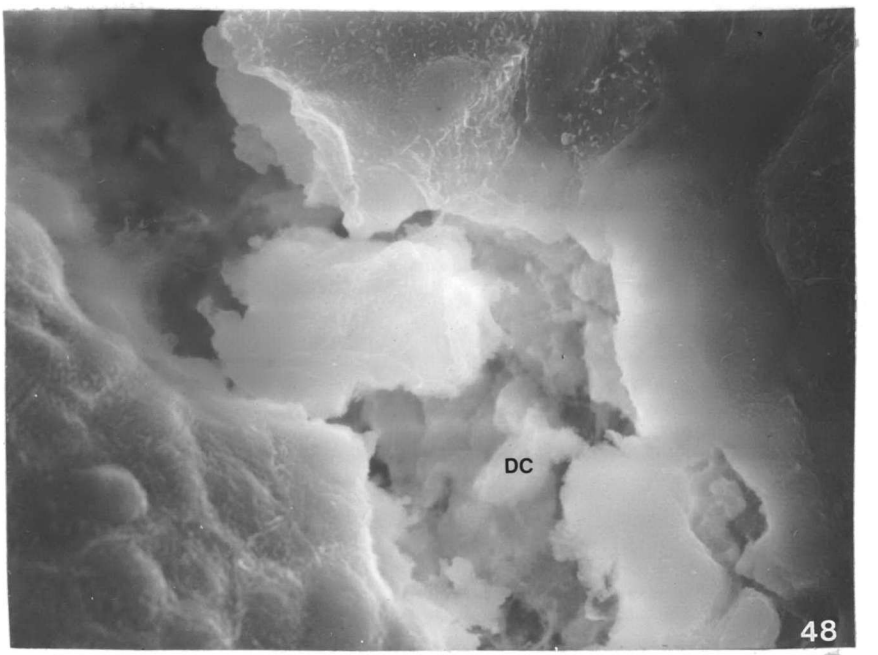
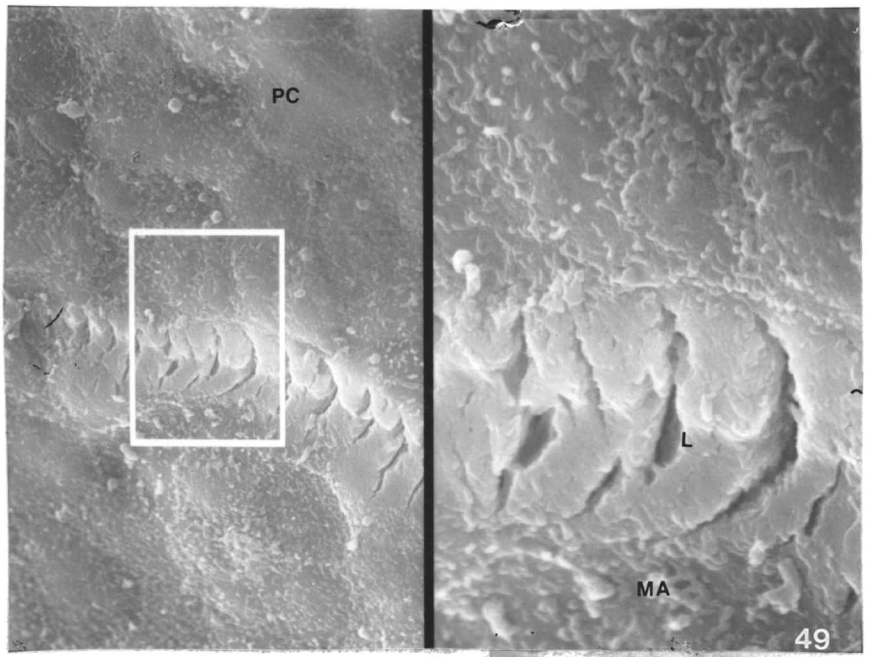

DC
48

Fig. 49. Scanning electron micrograph of skin from a 6½-day-old LACA-treated embryo. Peridermal (PC) skin lacerations (L), micro-appendages (MA). X 2,190

Fig. 50. Scanning electron micrograph of skin from a 6½-day-old LACA-treated embryo. Dermis (D), epidermis (E). Note the folding of the epidermis (arrows). X 220


PC
L
MA
49

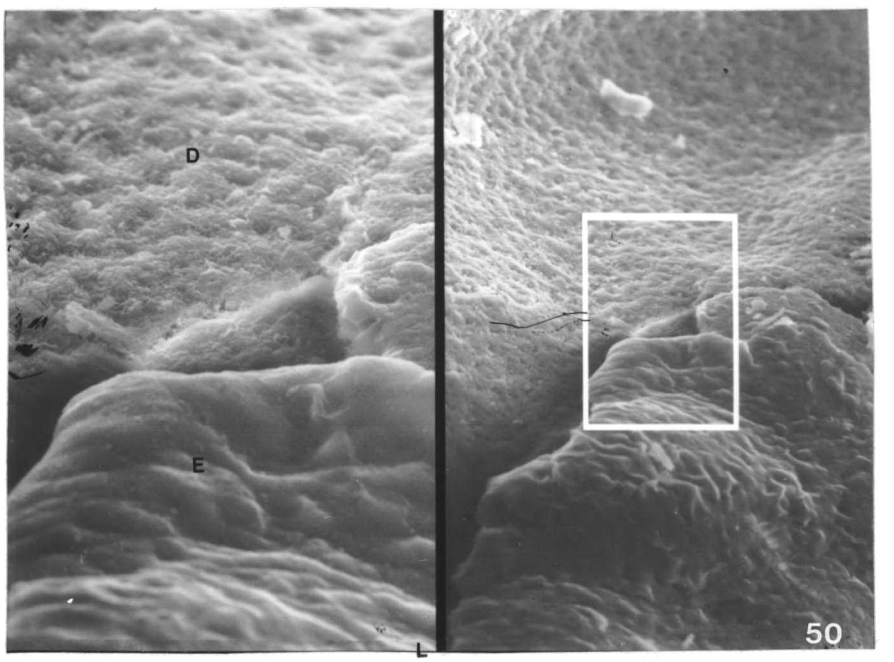
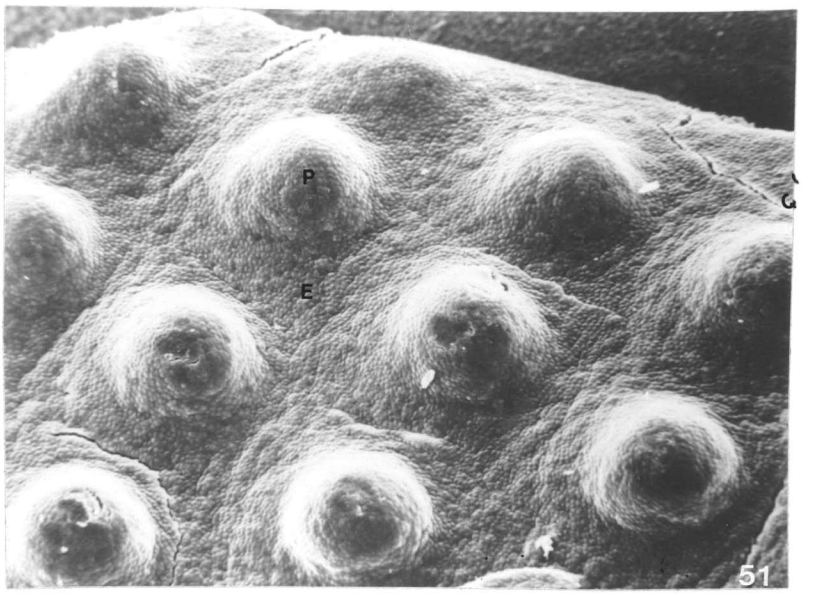

D
E
L
50

Fig. 51. Scanning electron micrograph of dorsal skin from 8-day-old saline-treated control embryo. Papillae (P) are well developed. Epidermis (E). X 119

Fig. 52. Scanning electron micrograph of dorsal skin from 8-day-old LACA-treated embryo. Note that the magnification of this micrograph is much higher than that of the control, but the papillae (P) are not as developed. X 570


P
E
51

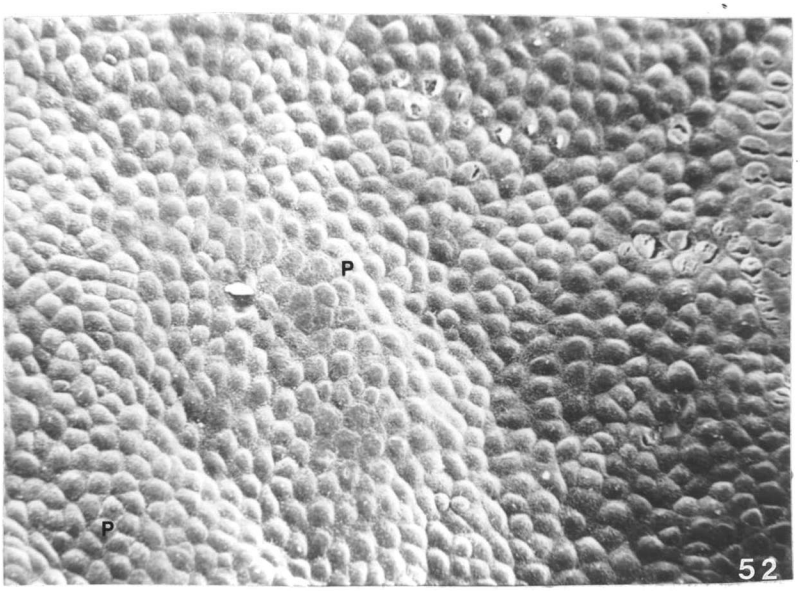
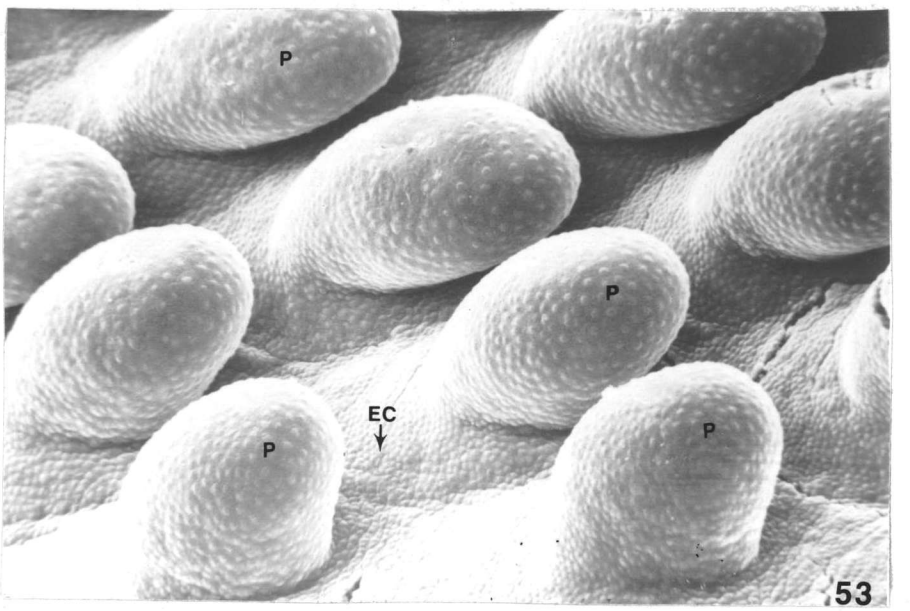
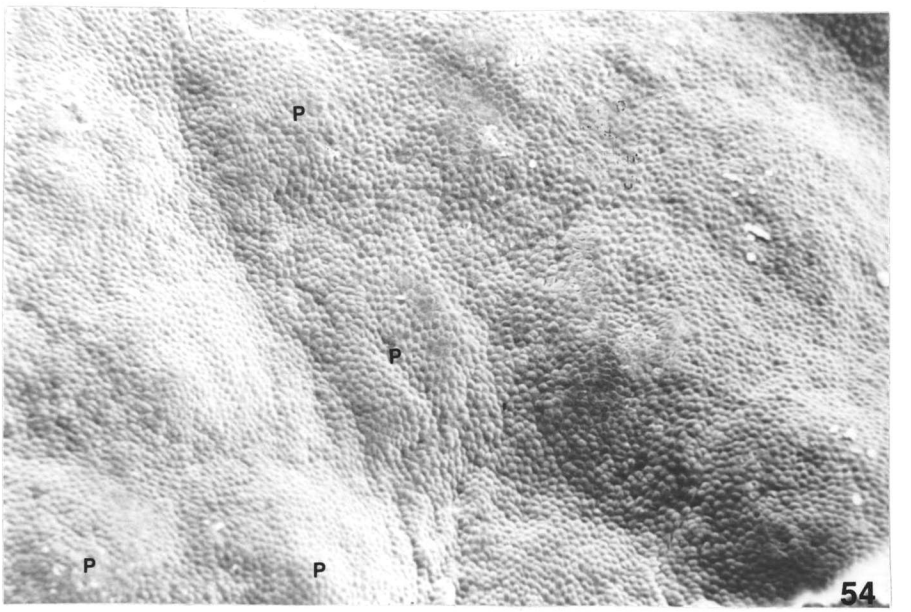

P
P
52

Fig. 53. Scanning electron micrograph of dorsal skin from 9-day-old saline-treated control embryo. Papillae (P), epidermal cells (EC), base of integument or epidermal surface (ES). X 175

Fig. 54. Scanning electron micrograph of dorsal skin from 9-day-old LACA-treated embryo. Note the inhibition of the dorsal papillae (P). X 175


P
P
EC
P
P
53


P
P
P
P
54

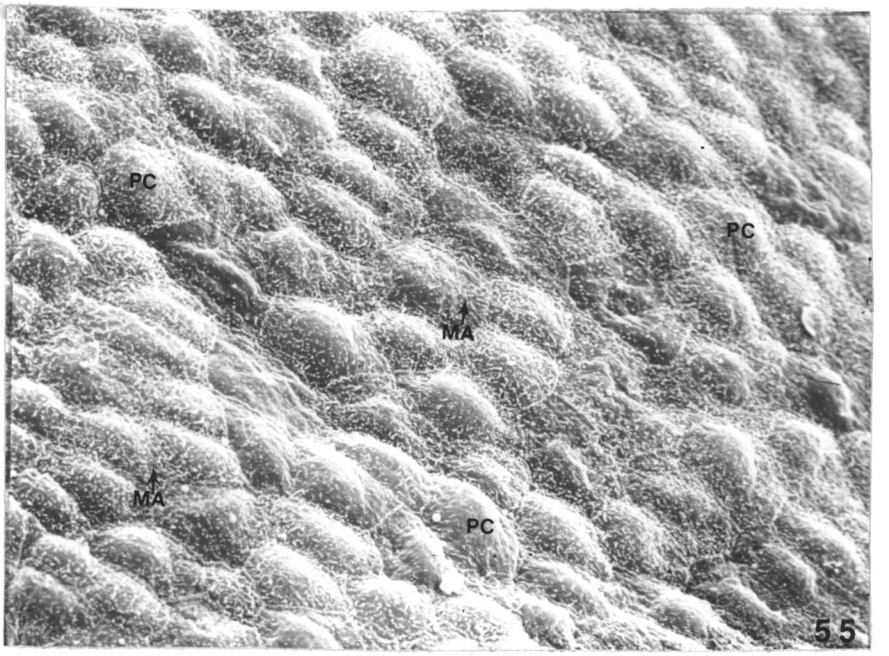
was suppressed by treatments with LACA. The outer epidermal cells of the skin from both the control and experimental embryos possessed abundant micro-appendages on their surfaces (Figs. 55-56). The epidermal cells of saline-treated (control) and analog-treated embryos are both comparable in size and shape at this stage during development. However, the membrane separating the individual cells was more apparent in the micrograph of the control as compared to that of the experimental (Figs. 55-56).

A cross-section of the control dermis through an area where papillae were not destined to form showed an abundance of extracellular fibrils (Fig. 57). Extracellular fibrils were not evident in a comparable section through the experimental dermis (Fig. 58). The arrangement of dermal tissue appeared disorganized and less compact.

As development proceeded, the shape and size of the control feather changed from 9 to 10 day. By the 10th day (Fig. 59) the feather began to narrow at the tip and base, but widened in the center as well as increased in length. However, this morphogenetic process did not take place in the 10-day-old experimental (Fig. 60). The development of feathers had not progressed beyond the feather germ stage. The 10-day-old experimental also exhibited what seemed to be a "blistering" of the epidermal sheath, between the existing feather germs. Figure 62 is a higher magnification of the "epidermal blister." This odd structure appeared to be void of dermal cells. The peridermal cells of the blister were similar in shape to the cells found on the adjacent papilla, but

Fig. 55. Scanning electron micrograph of epidermal surface between developing papillae of 9-day-old saline-treated control embryo. Note the peridermal cells (PC) and the extensive micro-appendages (MA). X 1,750

Fig. 56. Scanning electron micrograph of epidermal surface of 9-day-old LACA-treated embryo. Peridermal cells (PC) are evident with extensive micro-appendages (MA). These cells are indistinguishable from the peridermal cells of the control. X 1,750


PC
PC
MA
MA
PC
55

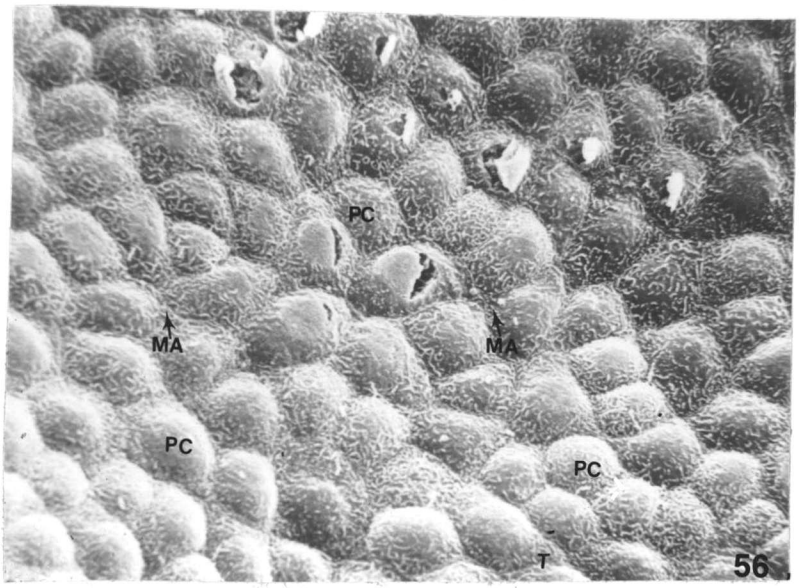
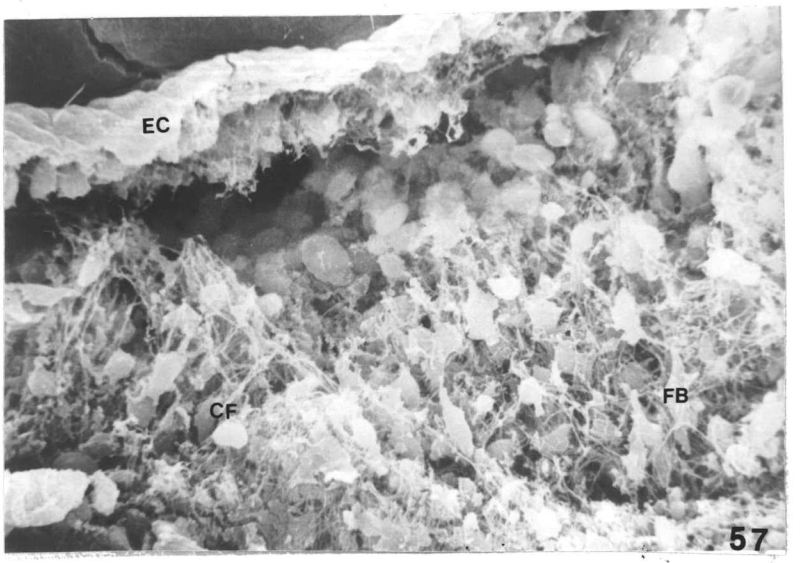

PC
MA
MA
PC
PC
T
56

Fig. 57. Scanning electron micrograph: cross section of 9-day-old dermis from saline-treated control embryo. Epidermal covering (EC), dermal fibroblasts (FB), collagen fibrils (CF). X 772

Fig. 58. Scanning electron micrograph: cross section of 9-day-old dermis from LACA-treated embryo. Epidermal covering (EC), no dermal fibroblasts or collagen fibrils are discernible, dermis (D). X 700


EC
CF
FB
57

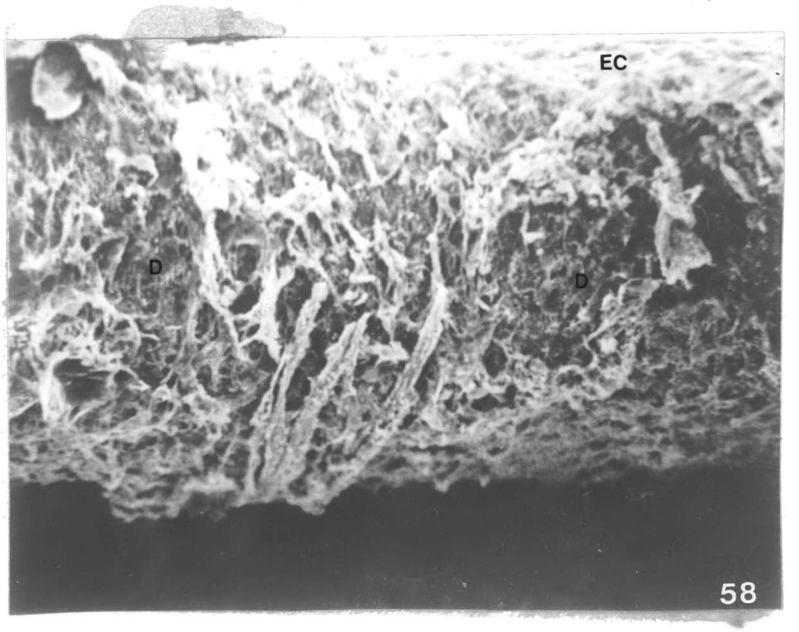
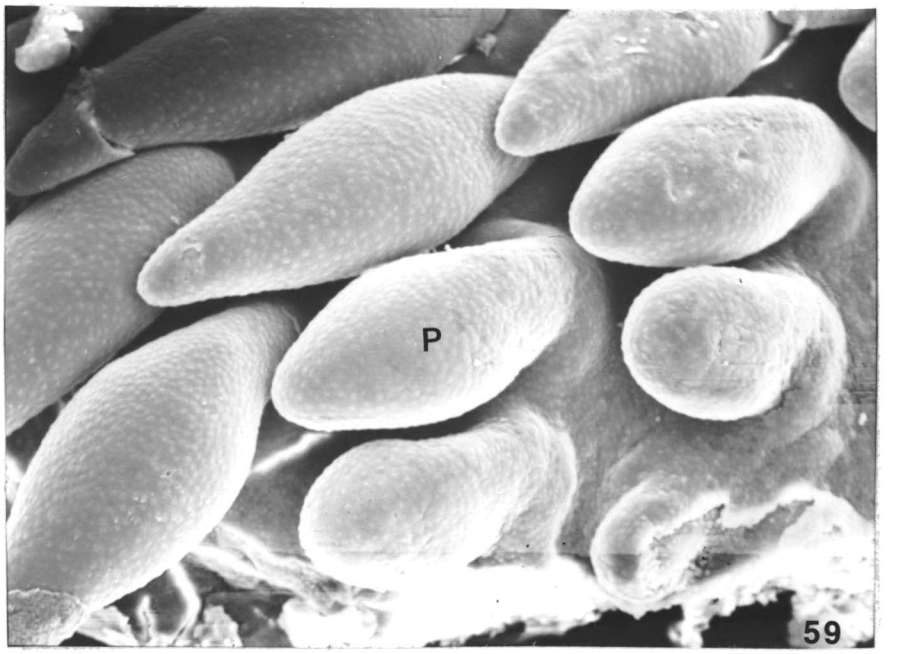

EC
D
D
58

Fig. 59. Scanning electron micrograph of dorsal papillae from 10-day-old saline-treated control embryo. Papillae (P). Note the distinct change in shape of the papillae at this age when compared to the papillae of the 9-day-old control. X 136

Fig. 60. Scanning electron micrograph of dorsal papillae from 10-day-old LACA-treated embryo. Papillae (P). Note the difference in size and shape of these papillae, compared to those in Fig. 59. Note also the presence of "epidermal blisters" (EB). X 136


P
59

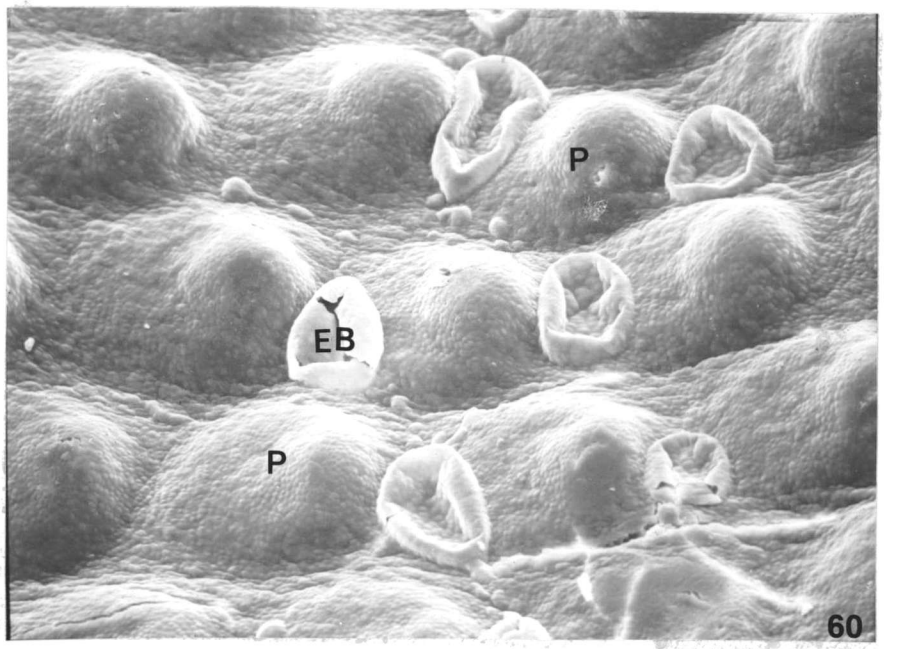
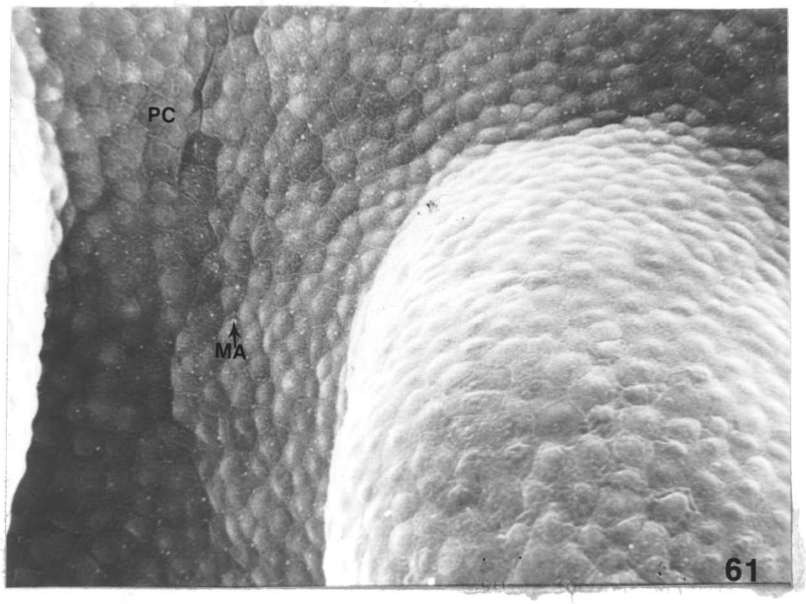
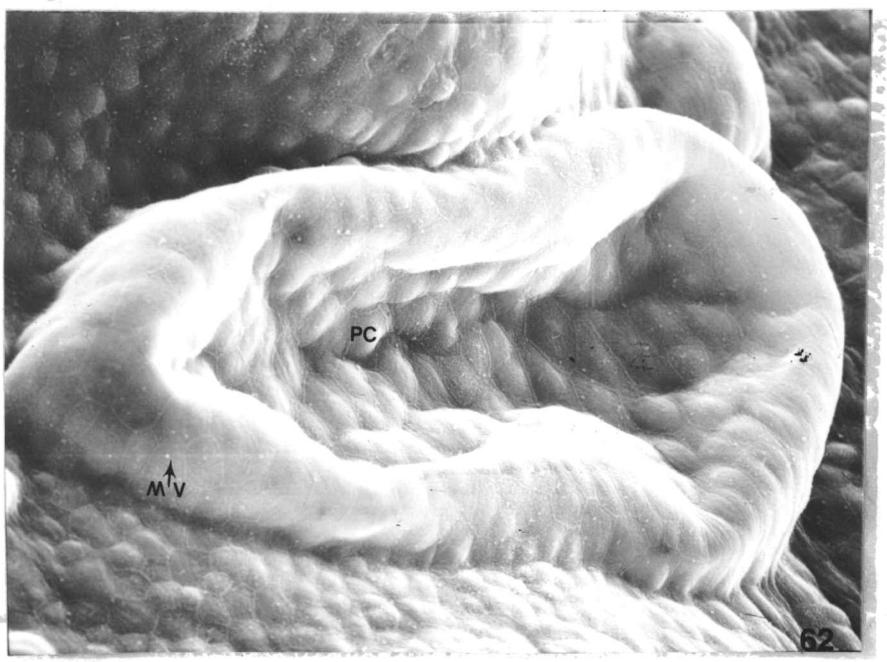

P
EB
P
60

Fig. 61. Scanning electron micrograph of epidermal surface between two developing papillae of the 10-day-old saline-treated control embryo. Papillae are indicated by large arrows, peridermal cells (PC), micro-appendages (MA). X 569

Fig. 62. Scanning electron micrograph of epidermal surface between two developing papillae of 10-day-old LACA-treated embryo. "Epidermal blister" indicated by arrows, peridermal cells (PC), micro-appendages (MV). X 658


PC
MA
61


PC
MV
62

were different in size; the "epidermal blister" cells seemed larger. Blistering was never apparent between the feather or papillae, as shown in a comparable micrograph of the control embryo (Fig. 61).

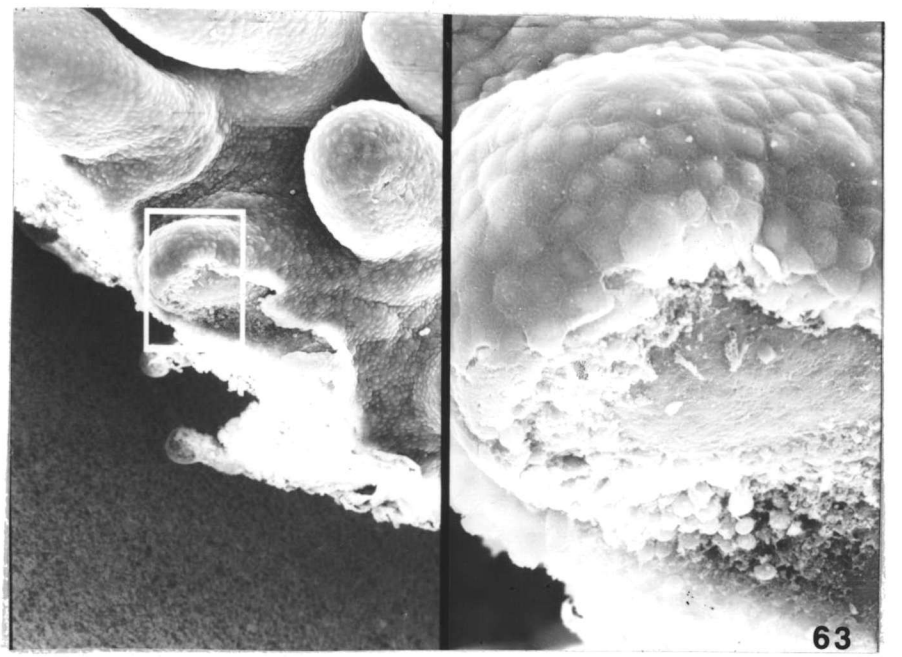
A longitudinal section of a dorso-lateral papilla from the 10-day control showed the distinct peridermal cells, the incipient barb vane ridge cells, and the highly vascularized dermal core (Fig. 63). This was the normal morphology of the feather after elongation started. The 10-day-old skin showed the compact arrangement of the dermal cells just underneath the dermis (Figs. 64-65). The epidermal sheath, composed of a single layer of peridermal cells (Fig. 64), was also evident in the 10-day-old control. Some fibrils were seen to be interwoven among the dermal fibroblasts of the 10-day-old control quite similar to the distribution of collagen fibrils found dispersed among the dermal cells of the 9-day-old control.

The micrograph in Fig. 66 represents a surface view of the extensive micro-appendages that were found on the peridermal cells of the 10-day-old experimentals. As mentioned earlier, these micro-appendages were not unique to the epidermal sheath cells of the LACA-treated embryos; they were also evident in the saline-treated control embryos (see Fig. 55).

The feathers of the 11-day-old controls were hardly distinguishable outwardly from those of the 10-day-old control (Fig. 67). The 11-day-old experimental (Fig. 68) exhibited feather suppression. No apparent development had taken place since the feather bud (hump stage), which

Fig. 63. Scanning electron micrograph: longitudinal section through papillae of 10-day-old saline-treated control embryo. Papillae (P), epidermal cell (EC), epidermal sheath (arrows), dermal cells (DC), and developing barb vane (BV). X 126

Fig. 64. Scanning electron micrograph: transverse section through dorsal skin of 10-day-old saline treated embryo. Dermal condensations (DC), papillae (P), epidermal sheath (ES), dermal fibrils (DF). X 84


63

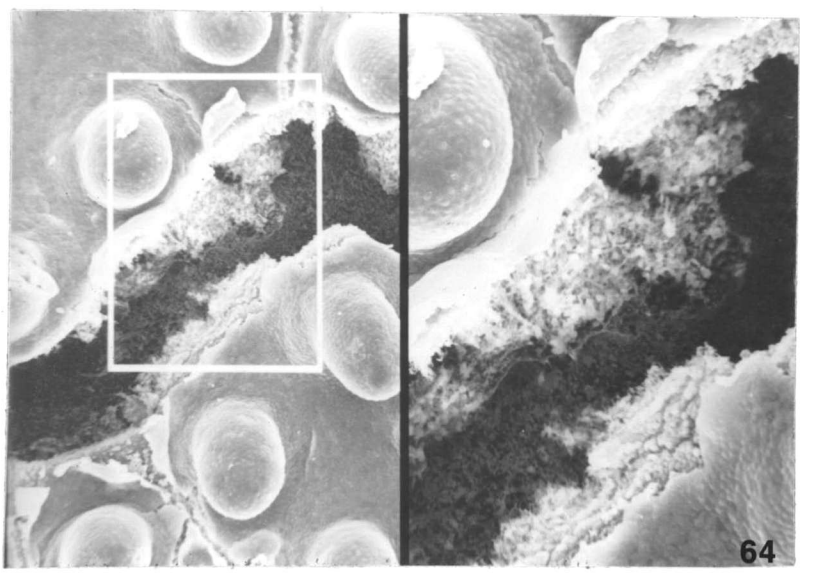
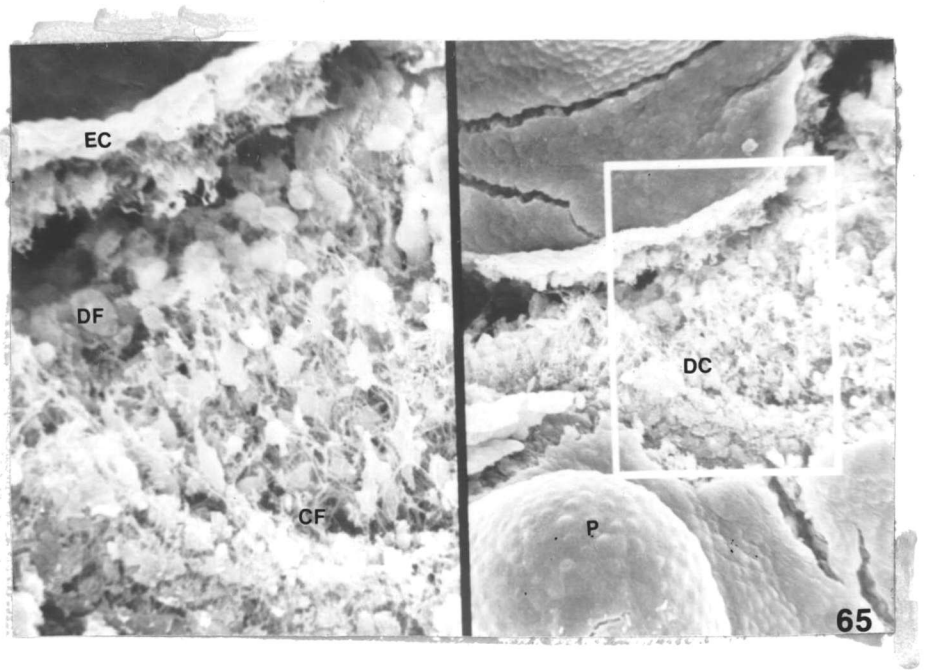

64

Fig. 65. Scanning electron micrograph: transverse section through dorsal skin of 10-day-old saline-treated control embryo. Papillae (P), ectodermal covering (EC), dermal condensations (DC), collagen fibrils (CF), dermal fibroblasts (DF). X 263

Fig. 66. Scanning electron micrograph of peridermal cells from 10-day-old LACA-treated embryo. Micro-appendages (MA), peridermal cells (PC). X 4,400


EC
DF
CF
DC
P
65

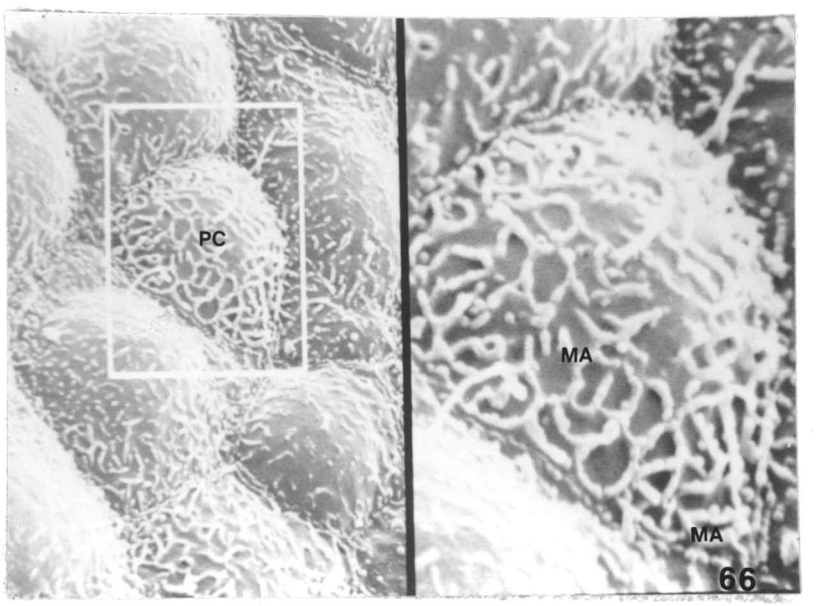
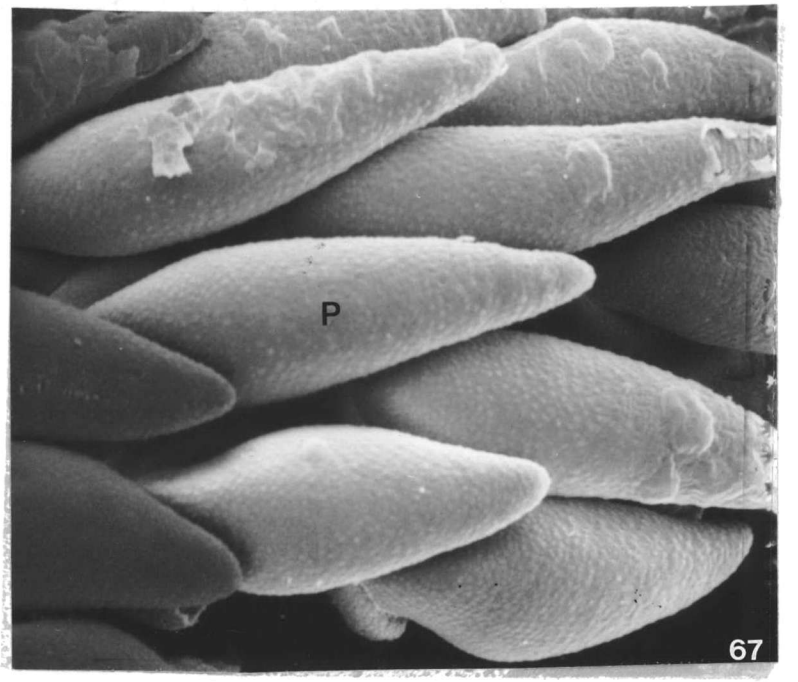
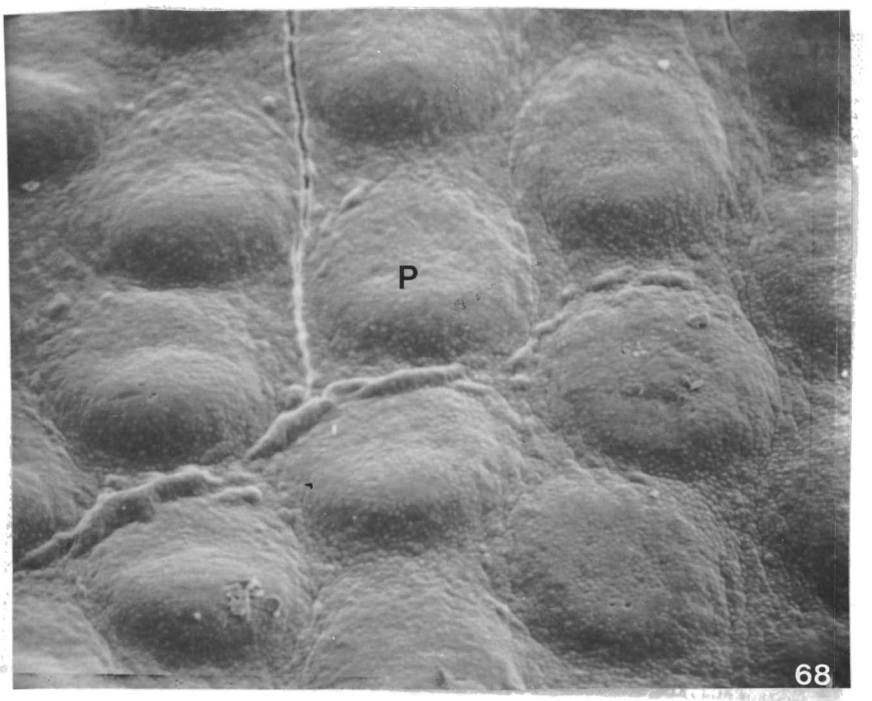

PC
MA
MA
66

Fig. 67. Scanning electron micrograph of developing feathers from 11-day-old saline-treated control embryo. Papillae (P); peeling of the ectodermal covering of the papillae is probably due to mechanical damage. X 100

Fig. 68. Scanning electron micrograph of developing papillae from 11-day-old LACA-treated embryo. Papillae (P); note the suppressed state of these papillae. X 100


P
67

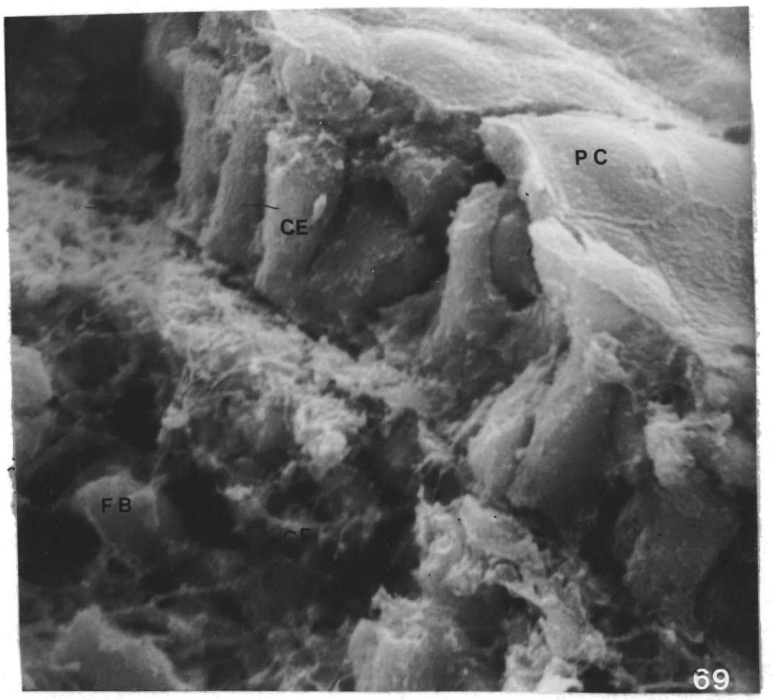

P
68

was usually characteristic of the 6-8-day-old control embryo. The "epidermal blisters" that were observed on the skin of the 10-day-old experimental (see Fig. 60) were also apparent on the skin of the 11-day-old experimental (Fig. 68). These blisters were also located in areas between developing papillae. Figure 69 (11-day-old control) shows the relationships between the flattened peridermal cells, the elongated columnar cells, and the basement membrane which lies between the basal cells of the epidermis and the dermis. Again the collagen fibrils were abundant throughout the dermis. The 11-day-old experimental (Fig. 70) showed few if any collagen fibrils in the dermis; they were not as defined as those seen in the control. Fig. 71 is another view of the epidermal surface between two developing feathers, but represents the 11-day-old control. The epidermal cells maintained their distinct squamous shape and no "epidermal blistering" was evident. On the other hand, Fig. 72 (11-day experimental) shows peeling of the epidermis on the surface of developing papillae. This peeling may be due to mechanical processing of the tissue for observation.

By the 12th day of incubation the control feathers have elongated extensively (Fig. 73). There was a distinct difference between the amount of growth in this control as compared to the feathers of the 12-day-old experimental (Fig. 74). Again the "epidermal blisters" were visible along the surface of the developing feather.

Fig. 69. Scanning electron micrograph: transverse section through dorsal skin of 11-day-old saline-treated control embryo. Peridermal cells (PC), columnar epithelial cells (CE), dermal fibroblasts (FB), and collagen fibrils (CF). X 1000

Fig. 70. Scanning electron micrograph: transverse section through dorsal skin of 11-day-old LACA-treated embryo. Peridermal cells (PC), ectodermal sheath (arrows), dermal condensations (DC). Note the lack of fibrils between the dermal cell condensations. X 965


PC
CE
FB
69

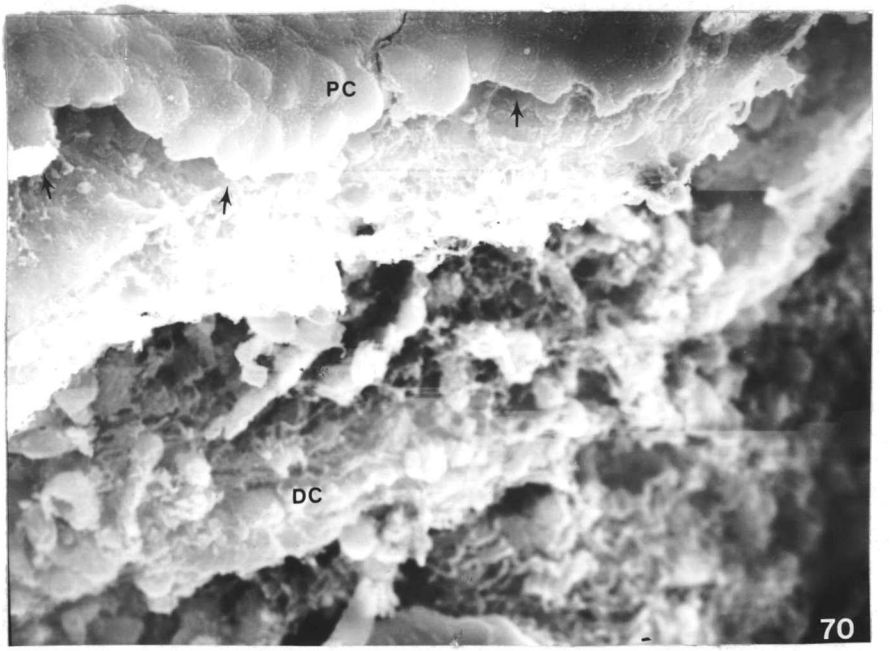
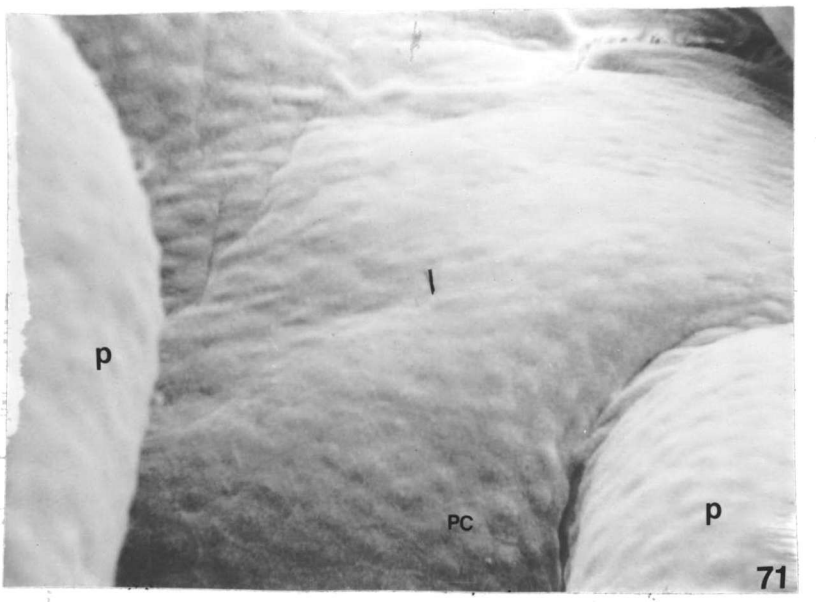

PC
DC
70

Fig. 71. Scanning electron micrograph of epidermal surface of 11-day-old saline-treated control embryo. Papillae (P), peridermal cells (PC). X 600

Fig. 72. Scanning electron micrograph of dorsal papillae from 11-day-old LACA-treated embryo. Papillae (P); the peeling of the ectodermal sheath is probably due to mechanical damage. X 600


p
l
PC
p
71

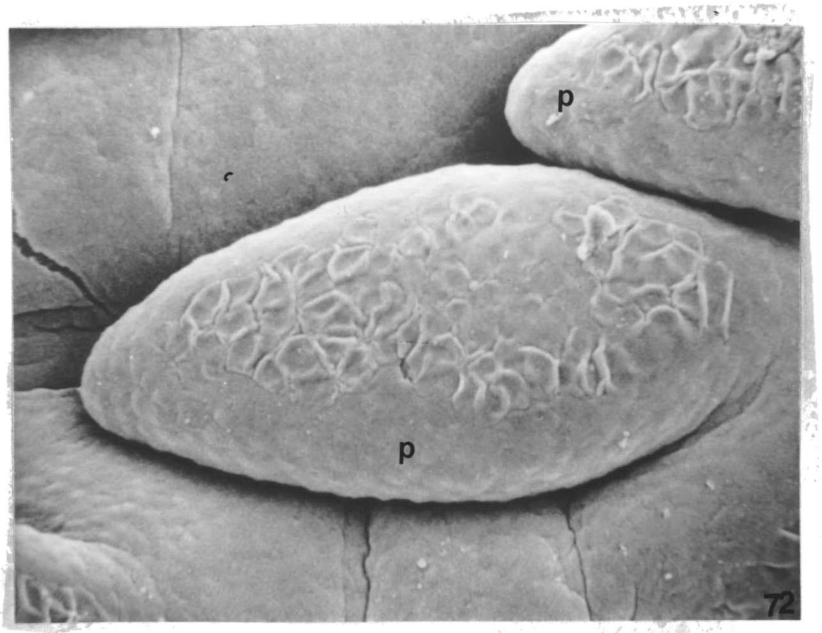
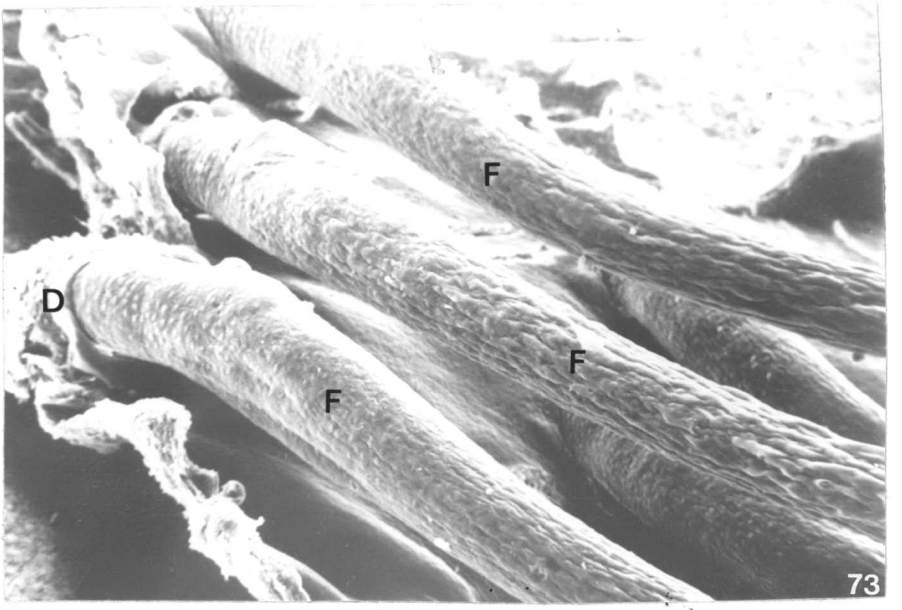
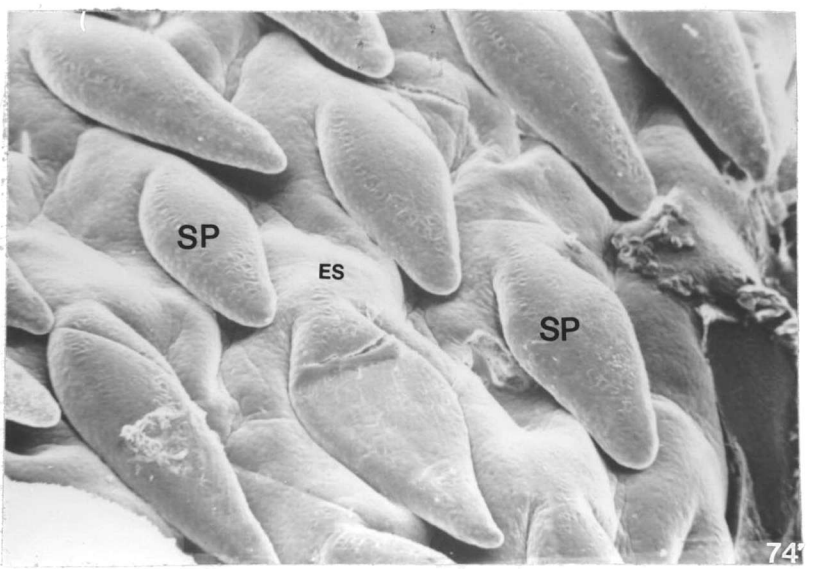

p
p
72

Fig. 73. Scanning electron micrograph of dorsal feathers from 12-day-old saline-treated control embryo. Feathers (F) have elongated (arrows) into cylindrical shaped structures; dermis (D). X 121

Fig. 74. Scanning electron micrograph of 12-day-old LACA-treated embryo. Suppressed dorsal papillae (SP), epidermal surface (ES). X 121


F
D
F
F
73


SP
ES
SP
74

A transverse section through a 13-day-old control feather (Fig. 75) illustrates the barb vane ridges and the centrally located dermal core. The flattened peridermal cells were distinct and normal in shape. The 13-day-old feather from the experimental embryo, at a comparable magnification, did not show these distinct cells. The epidermal surface seemed to be wrinkled or furrowed and did not resemble that of the control (Fig. 76). The growth and elongation of this 13-day-old feather did not seem to have been inhibited by treatment with LACA. Figure 77 represents another view of the characteristic flattened hexagonal peridermal cells of a control embryo.

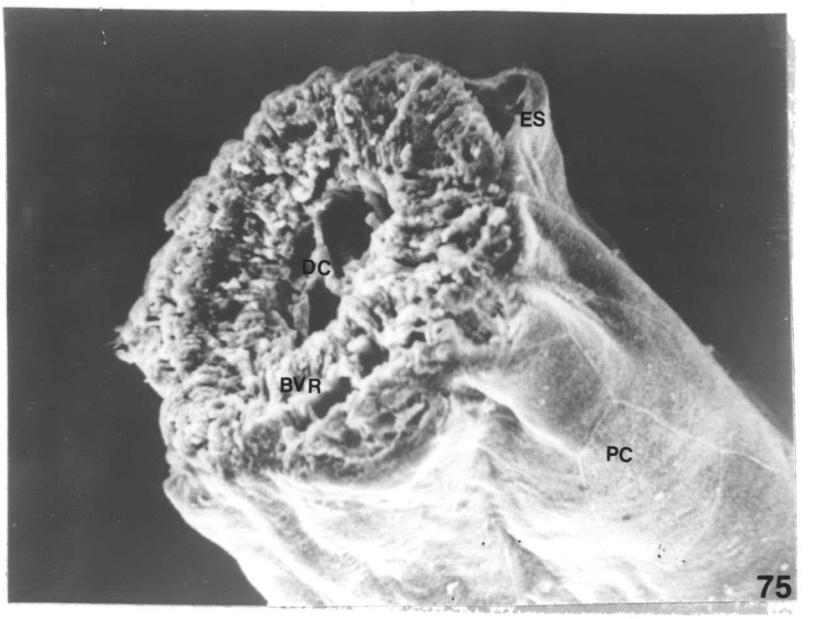
Transmission

Pericytes, Basal Cells, and the Epidermal-Dermal Junction

The epidermis at 6 days revealed two-cell layers: a layer of cuboidal basal cells and a flattened superficial cell layer called the periderm (Figs. 78-81). Figures 78 and 79 represent electron micrographs of 6-day-old control embryos and Figs. 80-81 represent electron micrographs of 6-day-old LACA-treated embryos. The pericytes of the epidermis both showed moderate to extensive micro-appendages (Figs. 78, 79,81) on the surface. The basal cells and the pericytes of both control and experimental contained similar types of cytoplasmic organelles such as mitochondria, endoplasmic reticulum, and ribosomes. The Golgi complex was not prominent in these epidermal cells. The basal cell of the 6-day-old showed a marked prominence of free ribosomes (Fig. 80). The basal lamina of the 6-day-old experimental was not

Fig. 75. Scanning electron micrograph: cross section of 13-day-old saline-treated control feather. Dermal core (DC), epidermal sheath (ES), developing barb vane ridges (BVR) and peridermal cells (PC). X 1000

Fig. 76. Scanning electron micrograph: surface view of feather from 13-day-old LACA-treated embryo. The peridermal cells (PC) are not apparent. Note the foldings of the epidermal sheath (arrows). X 1000


ES
DC
BVR
PC
75

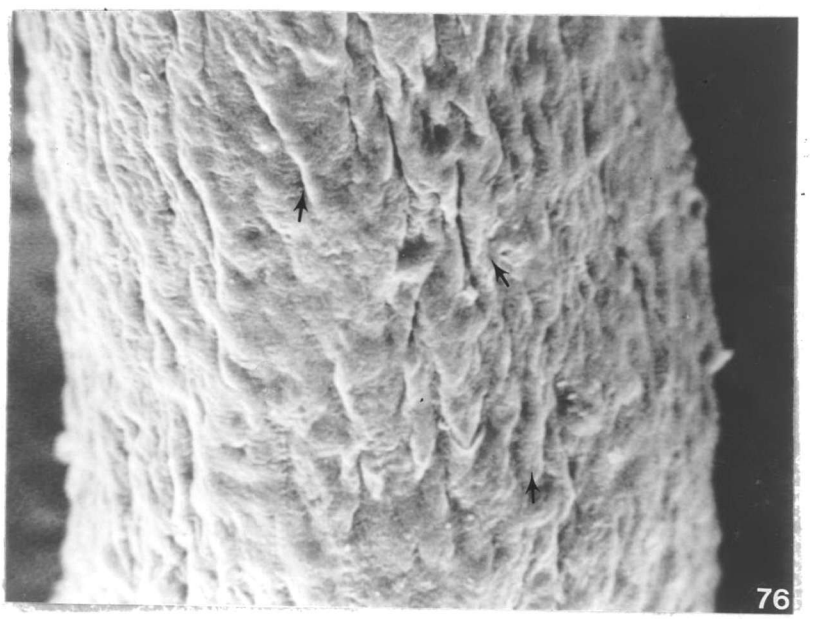

76

Fig. 77. Scanning electron micrograph of epidermal surface of 13-day-old saline-treated control embryo. Peridermal cells (PC); note the distinct hexagonal shape of the cells. X 3000

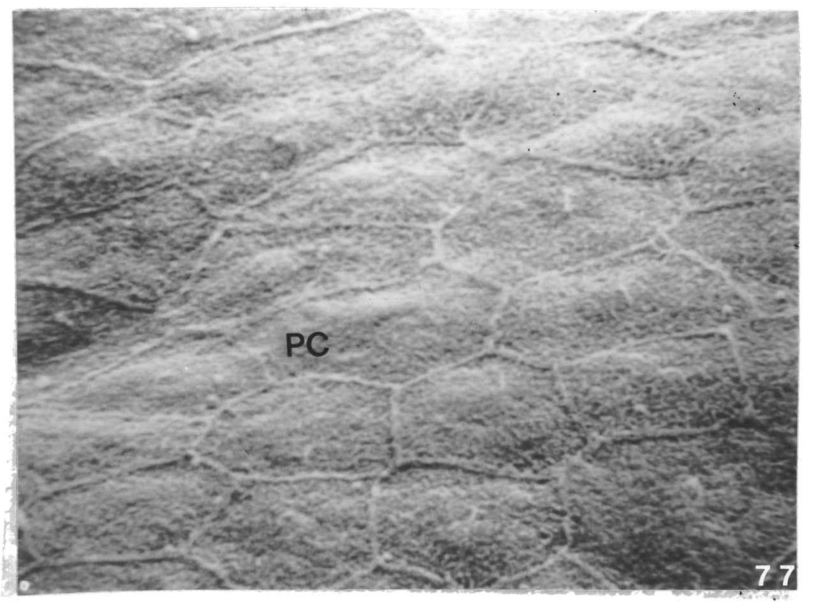
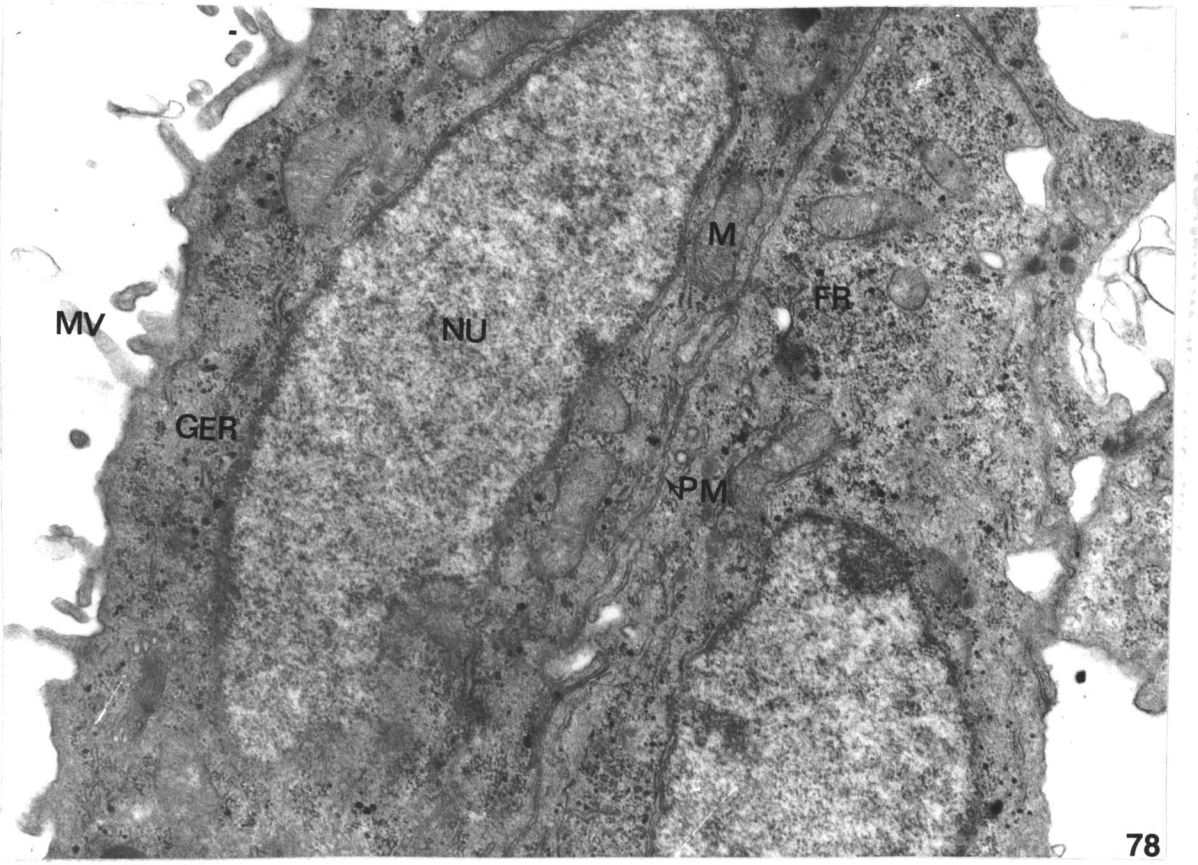

PC
77

Fig. 78. Transmission electron micrograph of peridermal cells from 6-day-old saline-treated control. Note the microvilli (MV), mitochondria (M), granular ER (GER), free ribosomes (FR). The plasma membrane (PM) and the nuclei (NU) are distinct. X 68,000

Fig. 79. Transmission electron micrograph of peridermal cells from 6-day-old LACA-treated embryo. The pericytes also have microvilli (MV) at the free surface. Mitochondria (M) are distinguishable, and scant granular ER (GER) are apparent. The plasma membrane (PM), nuclei (NU) and nuclear membrane (arrows) are distinct. X 68,000


M
FR
MV
NU
GER
PM
78

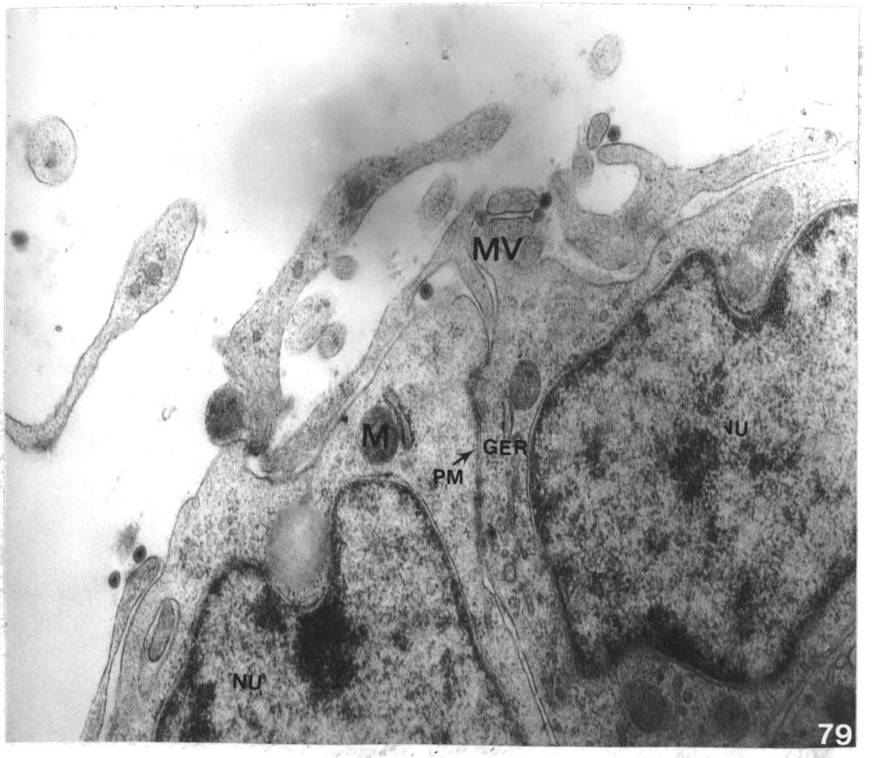
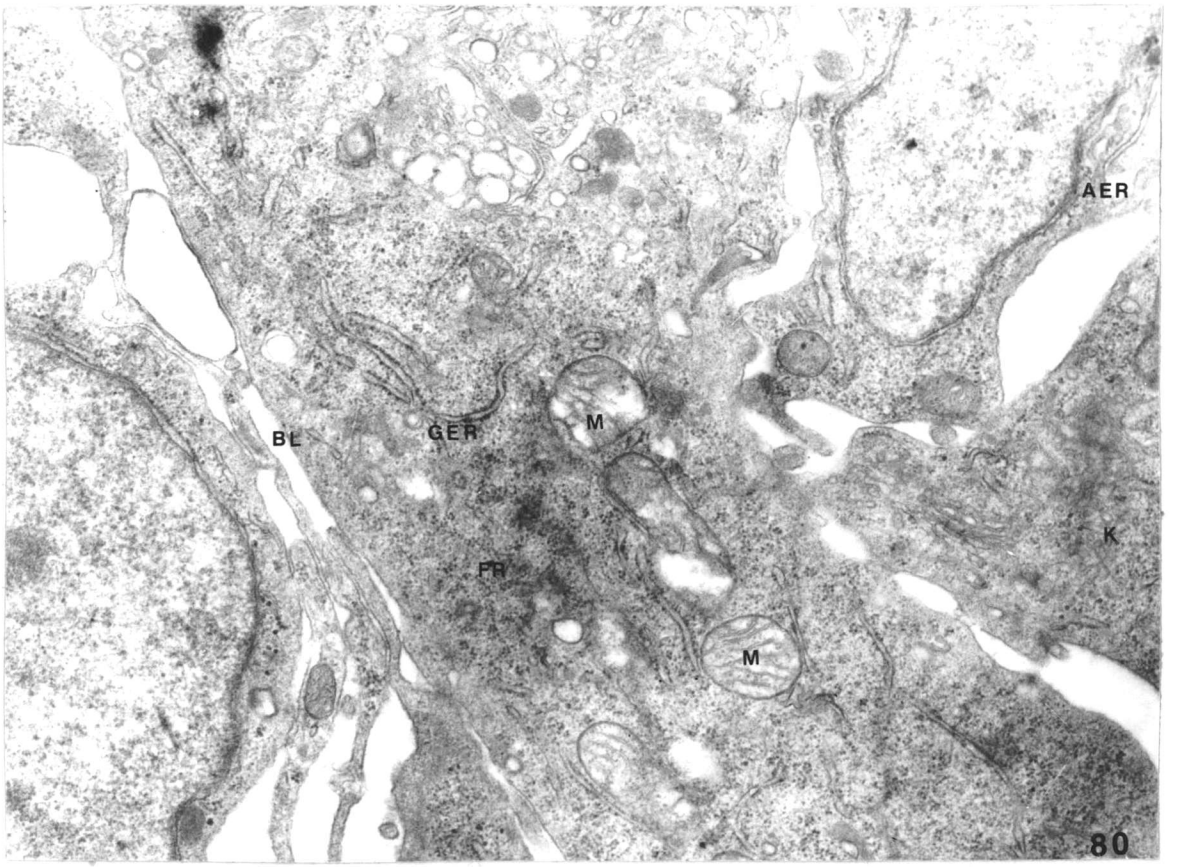
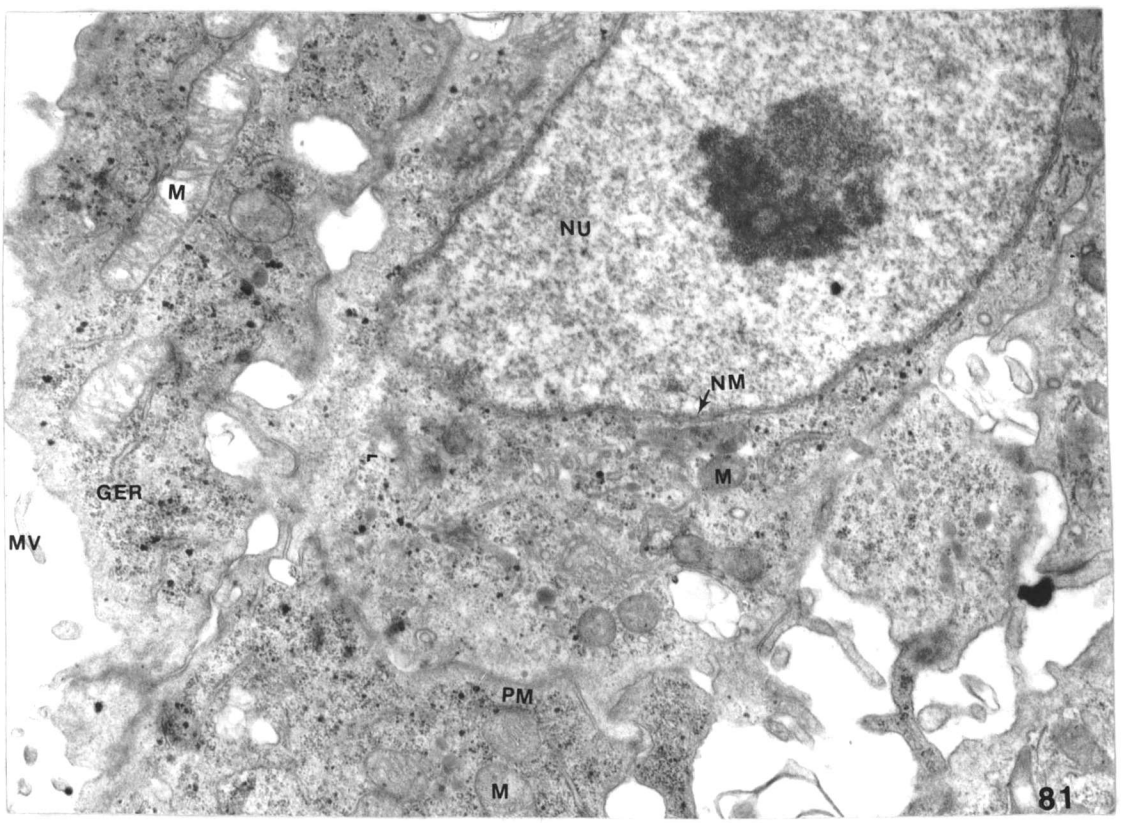

MV
M
GER
PM
NU
79

Fig. 80. Transmission electron micrograph of basal cells from 6-day-old LACA-treated embryo. Abundant mitochondria (M) are apparent with distinct cristae (arrow). Agranular (AER) and granular (GER) endoplasmic reticulum are also distinct. There is an abundance of free ribosomes (FR) in the basal cell. Note the basement lamina (BL) and absence of collagen fibrils underneath. X 68,000

Fig. 81. Transmission electron micrograph of peridermal cells from 6-day-old LACA-treated embryo. Note the microvilli (MV) and relatively large mitochondria (M) with cristae (arrows). The granular endoplasmic (GER) reticulum is barely visible. Nucleus (NU), plasma membrane (PM) and nuclear membrane (NM). X 68,000


AER
BL
GER
M
K
FR
M
80


M
NU
NM
M
GER
MV
PM
M
81

underlain by any apparent collagen fibrils or anchoring filaments that were usually found lying just underneath the normal 6-day-old basement lamina (Figs. 80,83). The continuous, relatively straight, basal lamina followed the contour of the basal cell cytoplasmic membrane (Figs. 82-92). The underlying mesenchyme is comprised of cells with many slender and broad cytoplasmic processes (filopodia) which were common to fibroblasts of both the control and experimental at nearly any age during development. This area of the chick embryo's integument has been called the epidermal-dermal junction. In the 6-day control (Fig. 82), the mesenchymal fibroblast cells contained an abundance of granular reticulum in tubular profiles. The Golgi complex was moderately prominent. Mitochondria were evident and their structure different from those found in the epidermis. The fibroblast's mitochondria were spherical and those of the epidermis were oval, elongated as well as spherical. Cross-sections of dermal cell cytoplasmic processes revealed thin microfilaments. Adjacent to the basal lamina and between mesenchymal cells collagen fibrils were found in the intercellular spaces. However, several of these findings were not apparent in the 6-day-old experimental (Fig. 83). Granular reticulum was not discernible nor were Golgi complexes. Small oval mitochondria were detectable. These mitochondria were similar in shape to those found in the epidermal cells. Microfilaments were not evident in the cytoplasmic processes and there was a scant amount of fibrils developed at the epidermal-dermal junction.

At nine days of incubation, the epidermal-dermal junction of the control integument appeared to be more organized (Fig. 84,86). The

Fig. 82. Transmission electron micrograph of the basement lamina (BL) at the epidermal-dermal junction of 6-8 day-old saline-treated control embryo. Note the abundance of collagen fibrils (CF) aligned just underneath the basement lamina in an orthogonal arrangement. The epidermal basal cells (BC) contain mitochondria (M) and granular ER (GER). The filopodia (FP) of the dermal fibroblasts are rich in granular endoplasmic reticulum (GER) and Golgi bodies (GB) are apparent. Collagen fibrils are also seen adjacent to the filopodia of the fibroblast cells. Extensive microfilaments (MF) are evident in cross sections of the filopodia. X 68,000

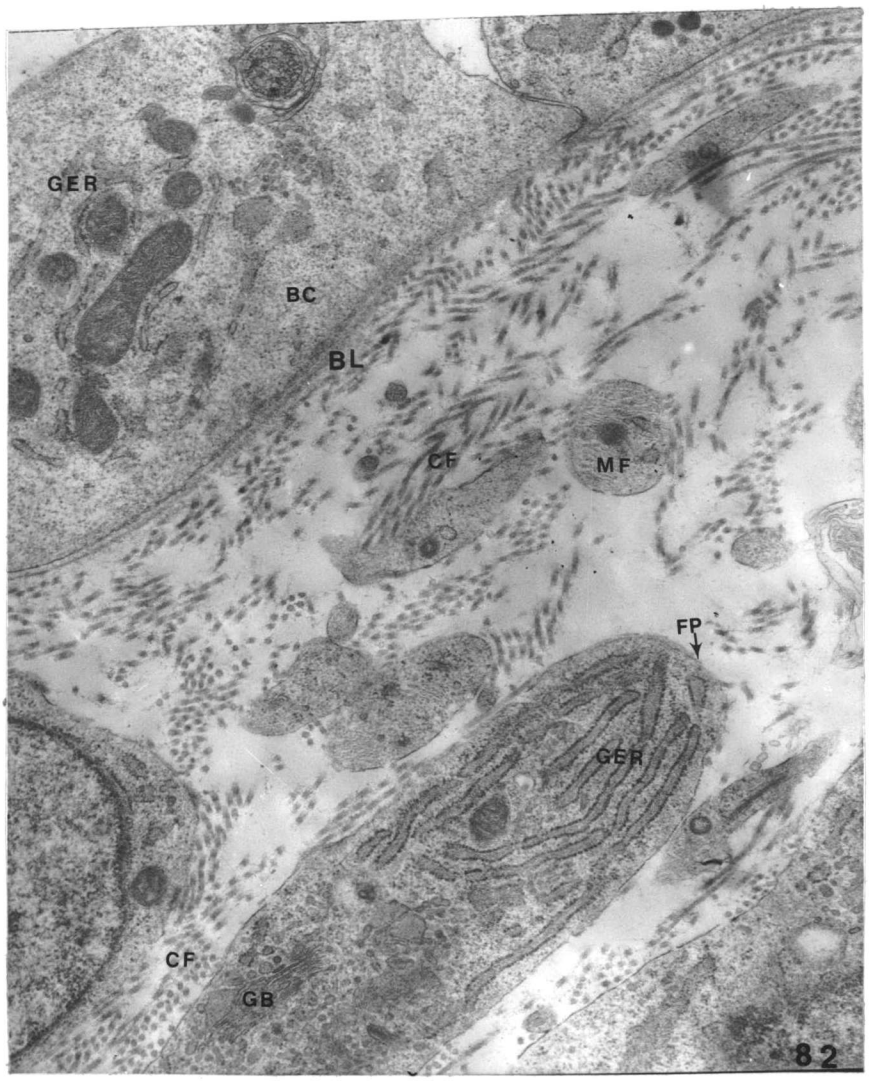

GER
BC
BL
CF
MF
FP
GER
CF
GB
82

Fig. 83. Transmission electron micrograph of the epidermal-dermal junction of the 6-8 day-old LACA-treated embryo. Note the lack of distinct collagen fibrils (CF) beneath the basement lamina (BL). The mitochondria (M) are visible in the basal cells (BC) of the epidermis. Scant amounts of granular ER (GER) are evident in these cells. There are no pronounced cellular organelles in the filopodia (FP) of the dermal fibroblasts; only the filopodia membranes (FPM) are apparent. X 64,600

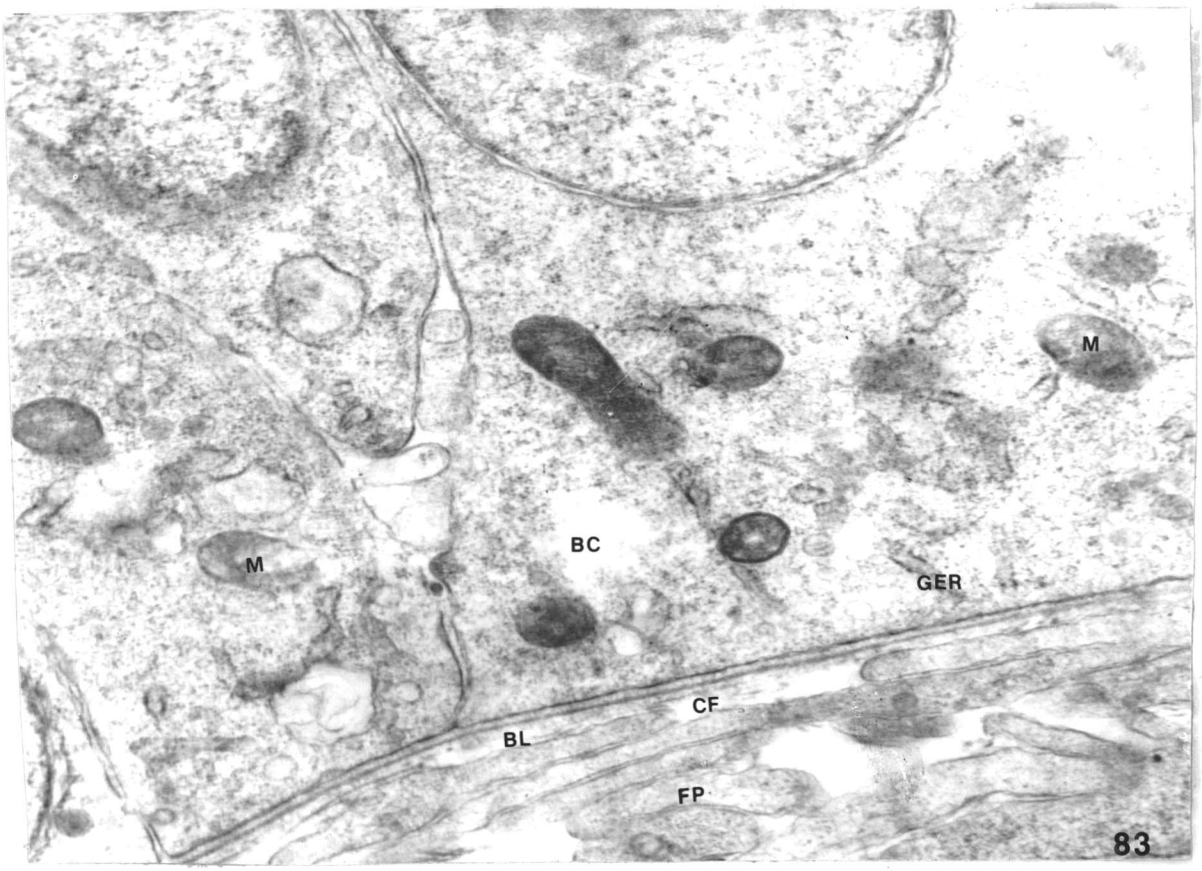
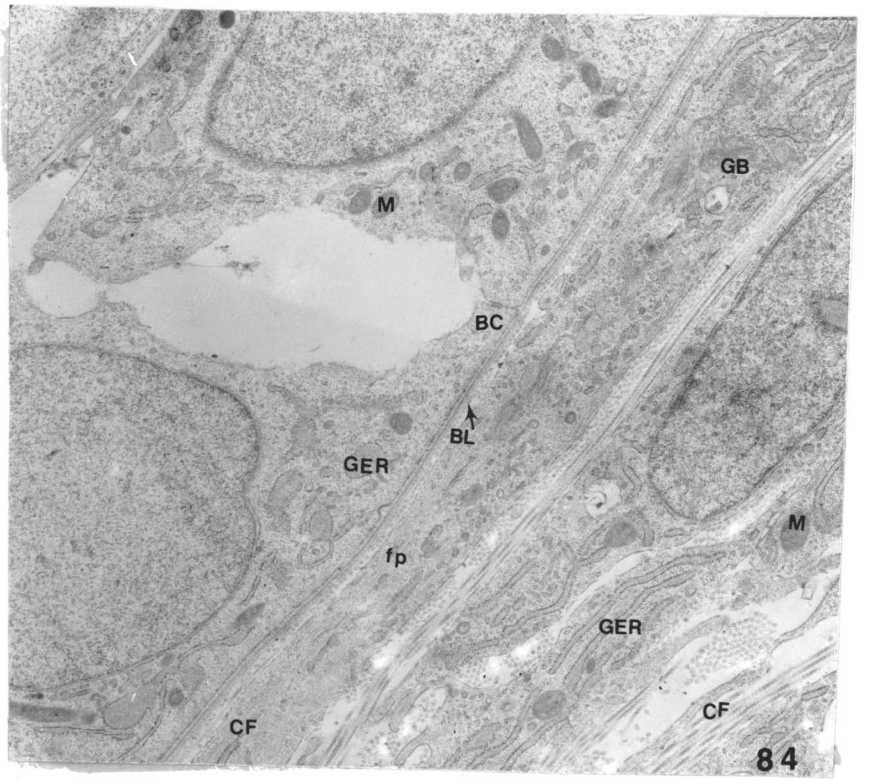

M
M
BC
GER
CF
BL
FP
83

Fig. 84. Transmission electron micrograph of the epidermal-dermal junction of skin from 9-day-old saline-treated control embryo. Collagen fibrils (CF) are aligned in an organized manner adjacent to the basement lamina (BL). Mitochondria (M) and granular ER (GER) are abundant in the basal cells (BC) and are even more extensive in the filopodia (FP) of the dermal fibroblasts. Note the arrangement of the collagen fibrils (CF) in the intercellular matrix. Golgi bodies (GB) are also extensive in the cytoplasmic extensions (filopodia). X 48,000

Fig. 85. Transmission electron micrograph of the epidermal-dermal junction of skin from 9-day-old LACA-treated embryo. Mitochondria (M) and granular ER (GER) are prevalent in the basal cells (BC). Collagen fibrils (CF) are present adjacent to the basement lamina (BL), but are not aligned in any organized or distinct manner; mitochondria (M) are evident in the filopodia. An interesting feature is the lack of compactness of the dermal fibroblasts beneath the basement lamina as seen in the control. X 48,000


GB
M
BC
BL
GER
M
fp
GER
CF
CF
84

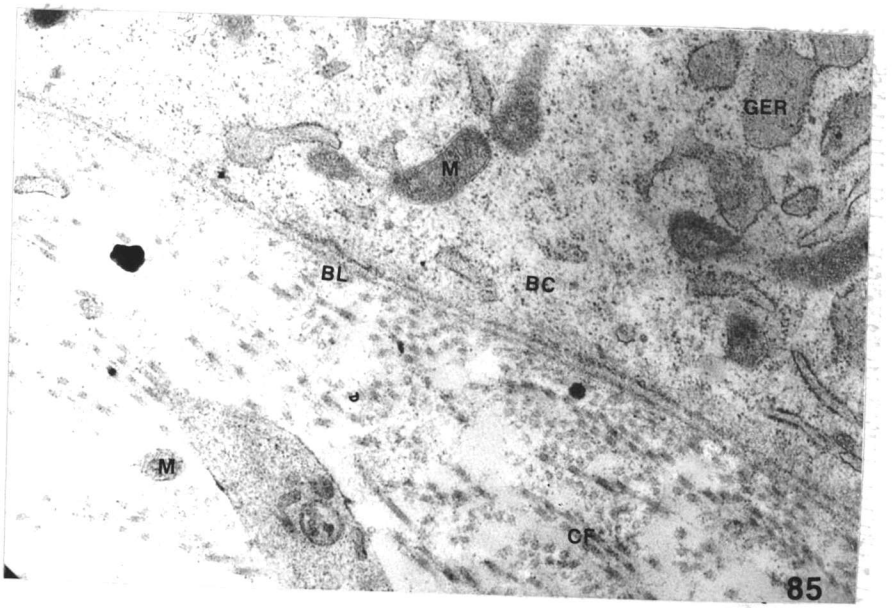

GER
M
BL
BC
M
CF
85

Fig. 86. Transmission electron micrograph of the epidermal-dermal junction of skin from 9-day-old saline-treated control embryo. This micrograph is a higher magnification of Fig. 84. Collagen fibrils (CF), basement lamina (BL), mitochondria (M), granular ER (GER), basal cells (BC), filopodia (FP), Golgi bodies (GB). X 68,000


GER
fp
M
fp
M
GB
CF
BC
BL
M
GER
86

Fig. 87. Transmission electron micrograph of epidermal-dermal junction of skin from 9-day-old LACA-treated embryo. The cellular organelles of the epidermal basal cells (BC) are faint. Some mitochondria (M) are present and the nuclei (NU) and nuclear membrane (NM) are pronounced. The basal lamina (BL) is prominent but the existence of collagen fibrils (CF) beneath it are apparent but are not as distinct or as abundant as they are in the 9-day-old control. Filopodia (FP) shows an abundance of microfilaments (MF). X 68,000

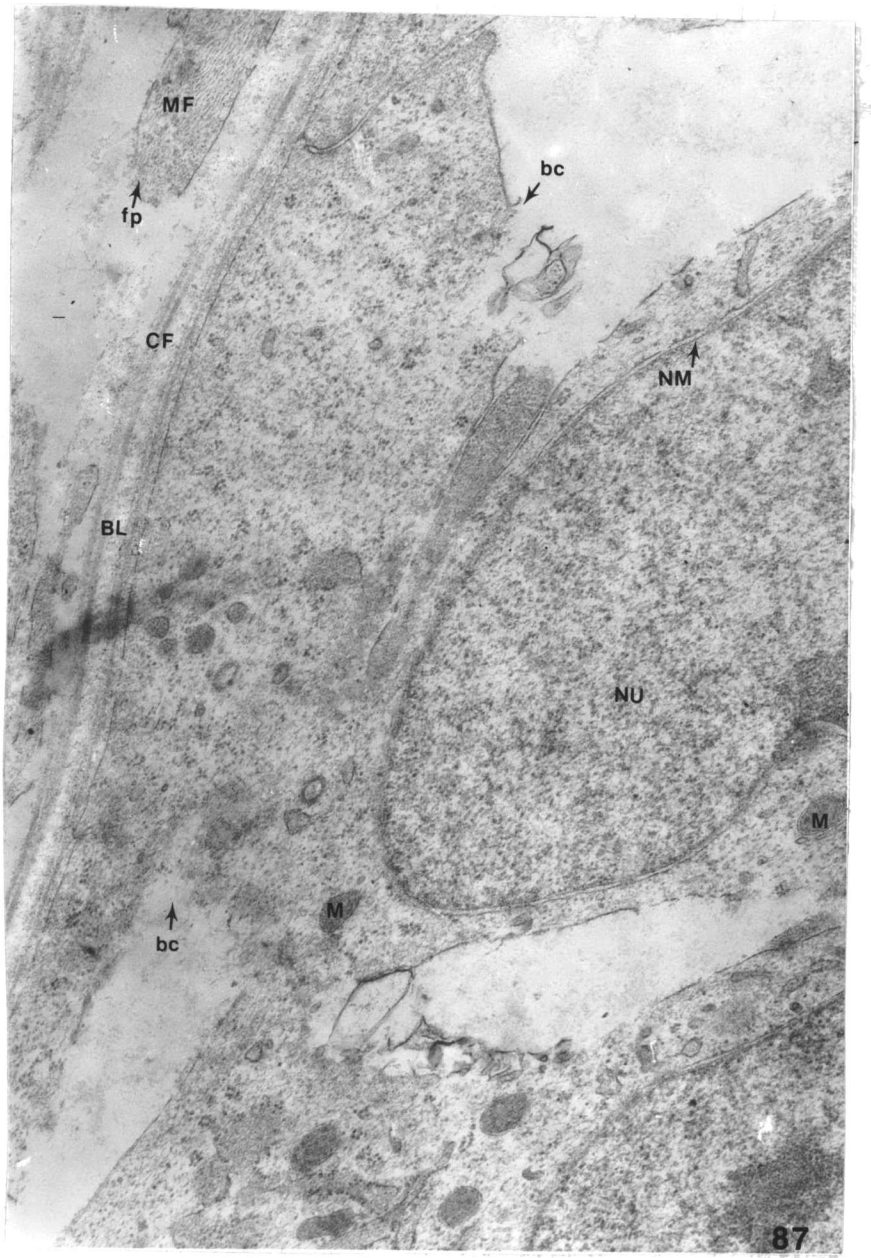

MF
fp
bc
CF
NM
BL
NU
M
M
bc
87

Fig. 88. Transmission electron micrograph of epidermal-dermal junction of skin from 12-day-old saline-treated control embryo. The basal cells (BC) show an abundance of granular ER (GER); mitochondria (M) are also evident. The basement lamina (BL) has collagen fibrils (CF) just underneath it, arranged in a lattice-like manner. The dermal filopodia (FP) contain an abundance of distinct Golgi bodies (GB), and microfilaments (MF). The granular endoplasmic reticulum (GER) is present in moderate amounts but mitochondria are not apparent. The collagen fibrils (CF) still maintain their lattice-like arrangement in the intercellular spaces of the dermal fibroblasts. X 140,000

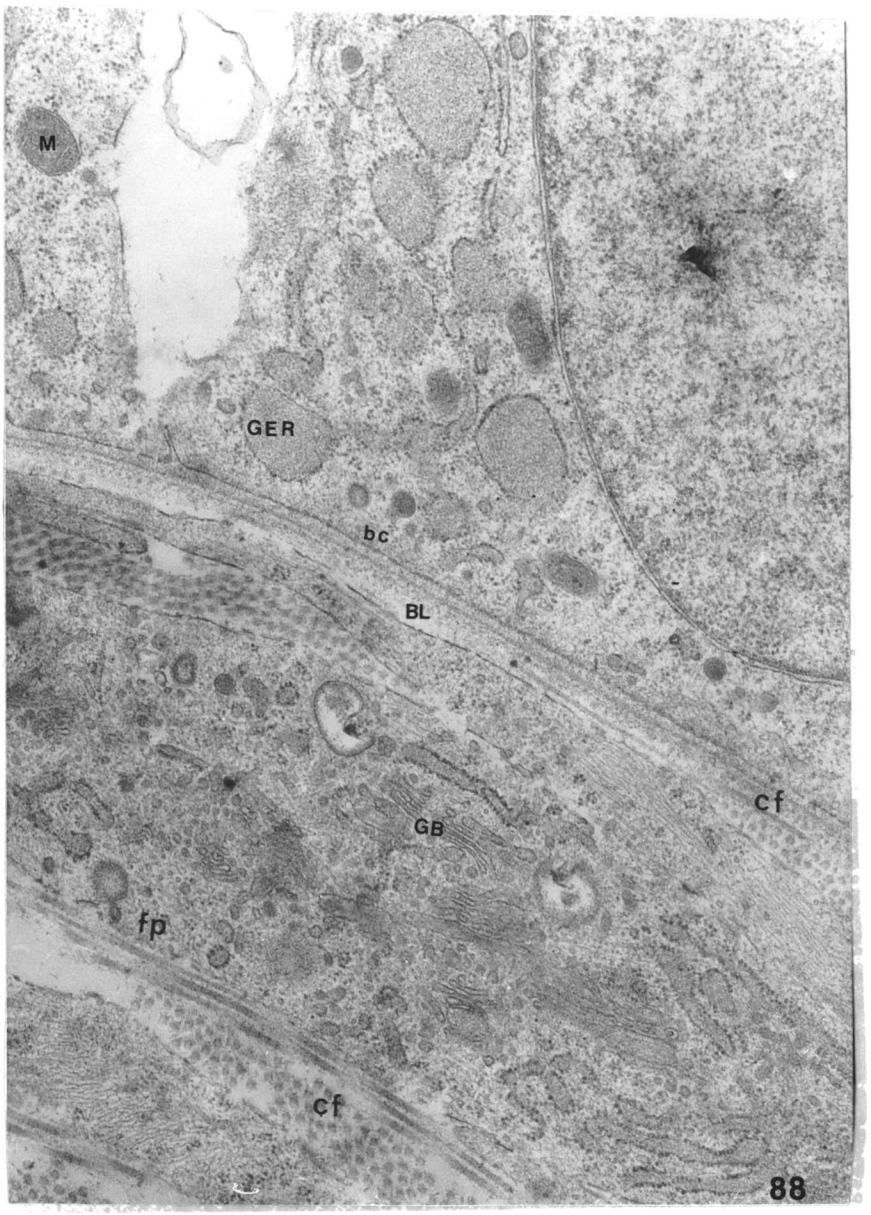

M
GER
bc
BL
GB
cf
fp
cf
88

Fig. 89. Transmission electron micrograph of epidermal-dermal junction of skin from 12-day-old LACA-treated embryo. Mitochondria (M) and granular endoplasmic reticulum (GER) are present in the basal cell (BC). Collagen fibrils (CF) are apparent beneath the basement lamina (BL) and are arranged in a lattice-like fashion, but the fibrils seem to be smaller than those of the control and less abundant. The Golgi bodies (GB) and mitochondria (M) are not evident in the filopodia (FP), a scant amount of granular ER (GER) is present. X 140,000

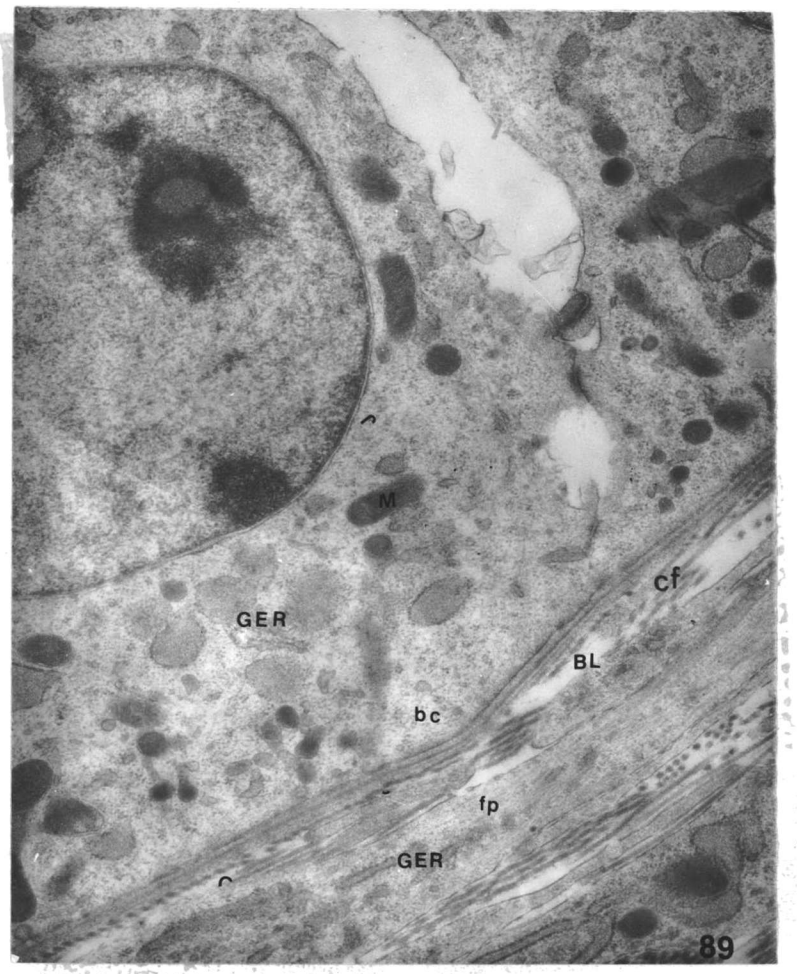

M
GER
cf
BL
bc
fp
GER
89

Fig. 90. Transmission electron micrograph of epidermal-dermal junction of skin from 14-day-old saline-treated control embryo. The basal cell of the epidermis contains mitochondria (M) and granular ER (GER). An abundance of collagen fibrils (CF) are orthogonically arranged just underneath the basement lamina (BL) and the extracellular matrix (EM). Granular ER (GER) and mitochondria (M) are abundant in the dermal cell filopodia. Golgi body (GB) is apparent. X 48,000

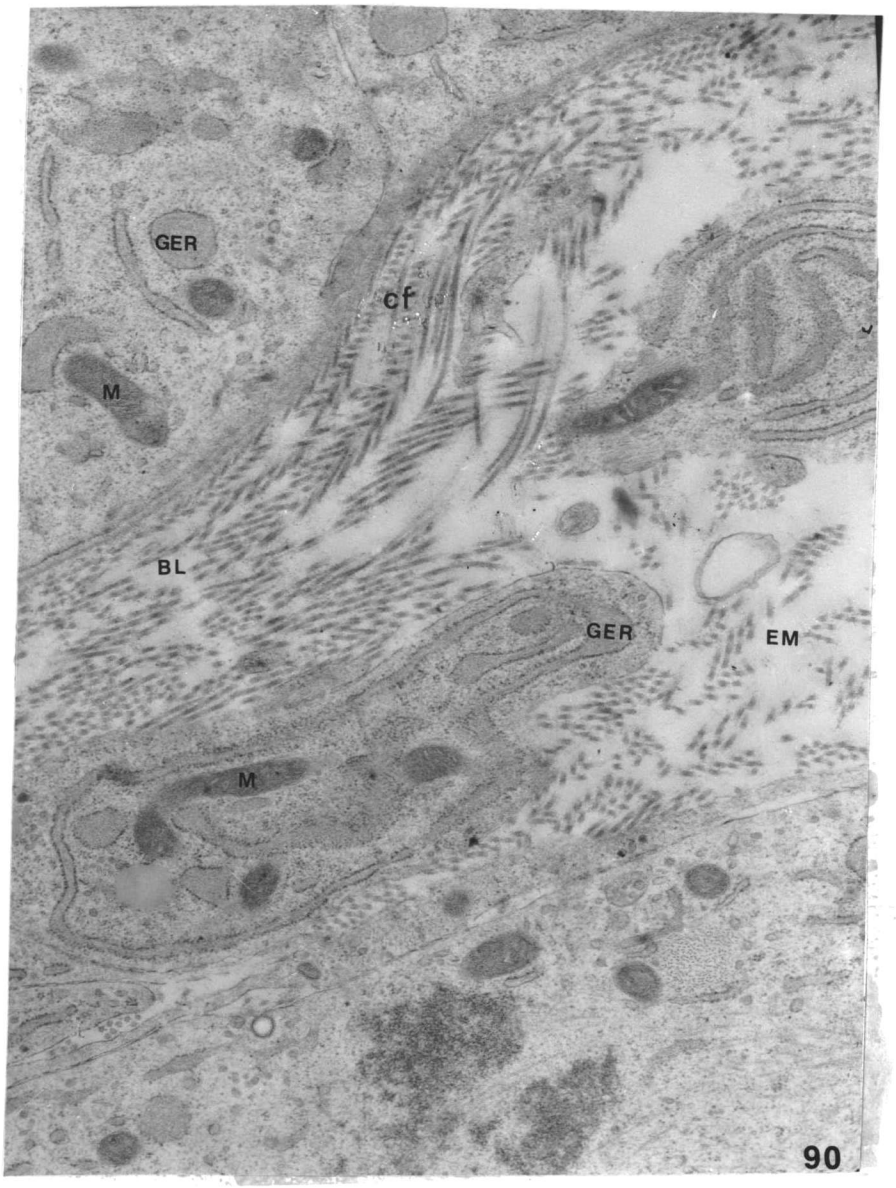

GER
cf
M
BL
GER
EM
M
90

Fig. 91. Transmission electron micrograph of epidermal-dermal junction of skin from 14-day-old LACA-treated embryo. The basal cells contain abundant mitochondria (M) and granular endoplasmic reticulum (GER) comparable to that of the control. The basement lamina (BL) is distinct, but the collagen fibrils (CF) beneath it are not aligned in an organized fashion. The filopodia (FP) contains mitochondria (M) and granular endoplasmic (GER). There is no compactness of the dermal cell filopodia. X 48,000

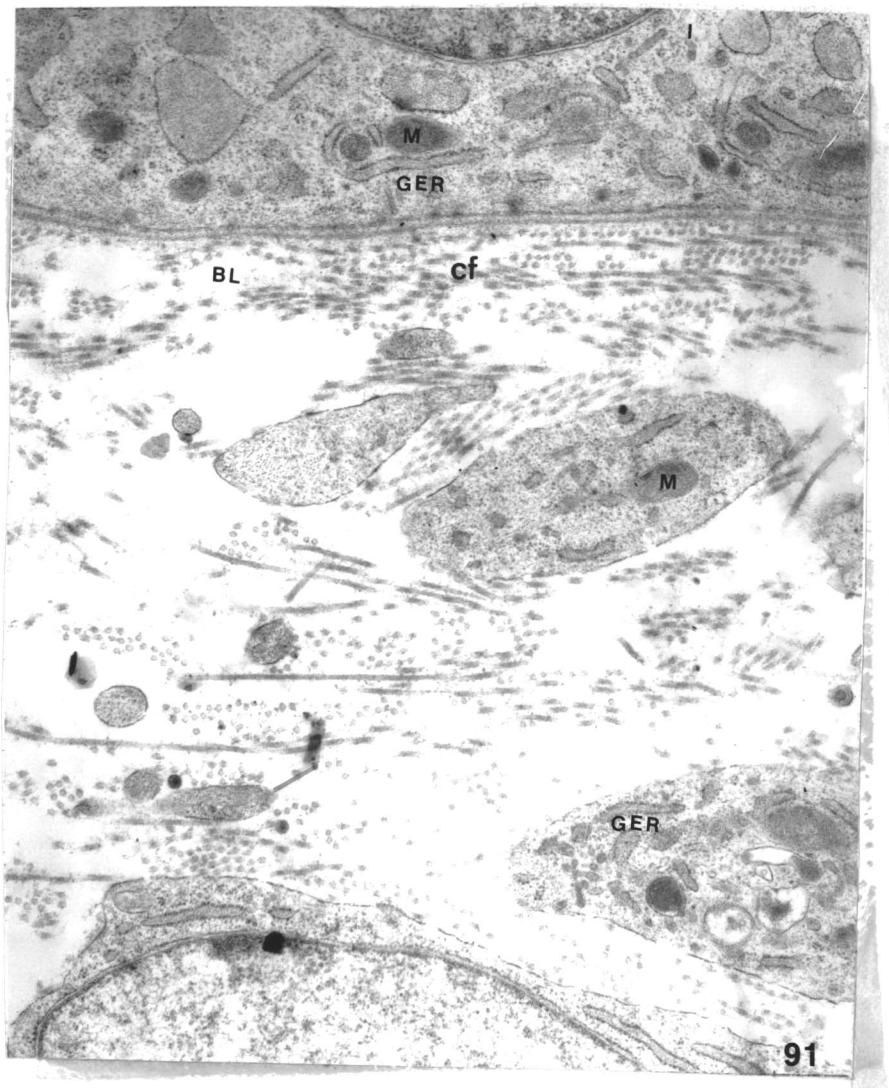
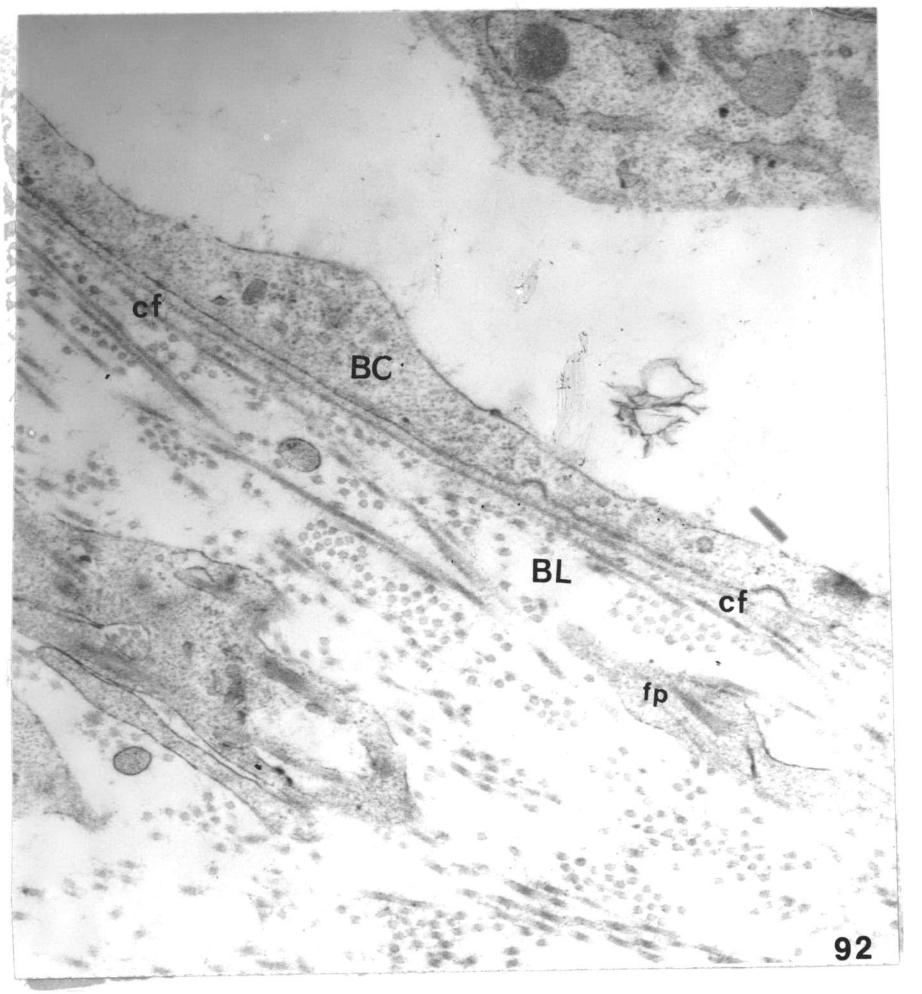

I
M
GER
BL
cf
M
GER
91

Fig. 92. Transmission electron micrograph of epidermal-dermal junction of skin from 14-day-old LACA-treated embryo. Note the irregularly shaped basal cell (BC) of the epidermis. The basement lamina (BL) is distinct but the arrangement of collagen fibrils (CF) is very unorganized. The filopodia (FP) of the dermal cell is quite sparse. No cellular organelles are distinct in the filopodia. X 48,000


cf
BC
BL
cf
fp
92

collagen fibrils were distributed more uniformly in the extracellular matrix. These fibrils were distinct, possessed an axial periodicity, and arranged in a prominent organized overlapping manner. The rough endoplasmic reticulum and Golgi complexes were numerous. The collagen was aligned adjacent to the basement membrane in a distinct arrangement. The 9-day experimental did not exemplify any organization at its epidermal-dermal junction. Normal fibrogenesis seemed to have been altered or inhibited (Figs. 85,87). The fibrils were detectable (Fig. 85) but loosely arranged; they were not detectable in Fig. 87. The mesenchymal cytoplasmic processes contained distinct mitochondria but no other organelles were obvious except microfilaments (Fig. 87). An interesting feature was the lack of compactness of these dermal cells just underneath the basement lamina and the lack of prominent cellular organelles in the basal cells of the epidermis.

As the development of the integument progressed to the 12th day, the epidermal-dermal junction of the control did not seem to become any more or less organized than the 9-day control (Fig. 88). It was possible to recognize beneath the basement membrane a collection of fiber bundles and filopodia of the dermal fibroblast. The filopodia were usually recognized by their extensive cytoplasmic elements. In comparison to the 9-day control, the number of Golgi increased within the filopodia of the 12-day control, possibly indicating that fibrogenesis was progressing at a much faster rate. The granule studded reticulum was still abundant but, oddly enough, the mitochondria were

not distinct. The cytoplasmic processes of the 12-day-old control contained numerous microfilaments. The epidermal-dermal junction of the 12-day-old experimental (Fig. 89) was somewhat more organized than the 9-day experimental, but less organized than the 12-day-old control. The basement membrane was distinct. Collagen fibrils were evident beneath the membrane. Cytoplasmic extensions were apparent but their inclusions were barely visible. Granular endoplasmic reticulum was present but appeared very faint; the other organelles were not visible. The fibrils of both control and experimental seemed to have an axial periodicity and were both aligned in an overlapping manner, but the fibrils found in the control were more abundant.

The fibrils underneath the basement lamina and in the extracellular space of the 14-day-old control were arranged orthogonally (Fig. 90). The cytoplasm of the fibroblasts was characterized by the presence of profiles of a large number of elongate and spherical elements of endoplasmic reticulum. These were bound by a membrane which owes its density to a large accumulation of ribosomal granules on its outer matrix surface. The mitochondria were abundant; Golgi and microfilaments were present but few in number. Figures 91 and 92 are electron micrographs of the epidermal-dermal junctions of 14-day-old experimentals. A striking feature was the unorganized manner in which the collagen fibrils were deposited underneath the basement membrane and within the intercellular spaces. Golgi bodies were not apparent; mitochondria were scant; and the endoplasmic reticulum was not elongated as in the

controls. There was some irregularity in the arrangement of the epidermal cells (Fig. 92). Normally the basal cells were columnar in shape, extending from the basement lamella upward to the pericytes, as seen in Figs. 83,84,86,88,89, and 90. But the basal cell in this particular micrograph seemed to be a flattened, elongated cell similar in structure to the filopodia of the mesenchymal cells.

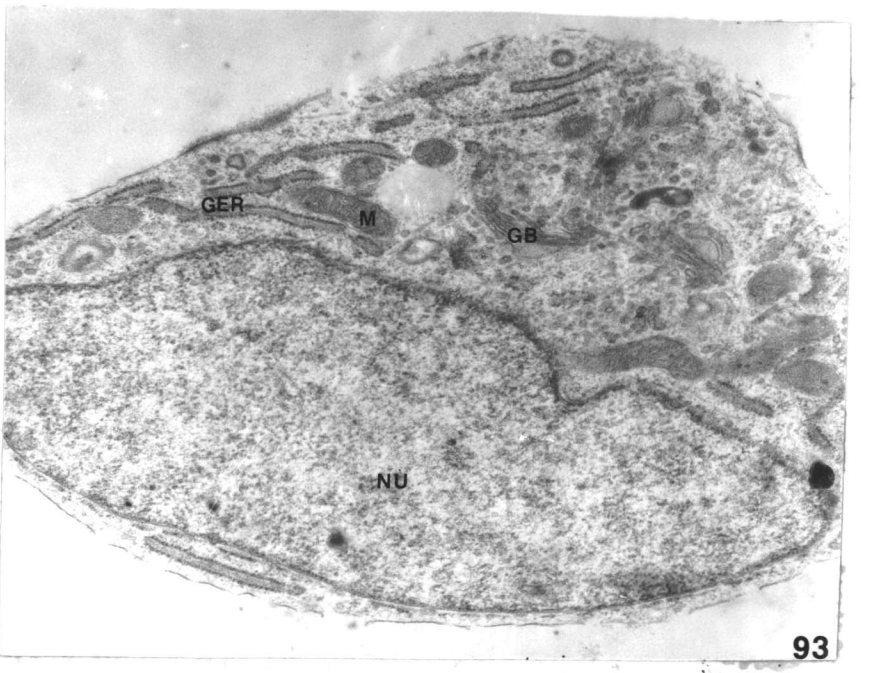
Dermal Fibroblast

The dermal fibroblast of the 5-day-old control embryo showed many distinct organelles such as granular endoplasmic reticulum, mitochondria, and Golgi bodies. The plasma membrane of the control contained numerous breaks (Fig. 93). The fibroblasts from the dermis of the embryos treated with LACA was somewhat similar in morphology to the control, except that Golgi bodies were not apparent. The attached ribosomes, endoplasmic reticulum and mitochondria were present but not as abundant (Fig. 94). An interesting feature of this experimental cell was that small deposits of collagen fibrils were discernible just outside the plasma membrane.

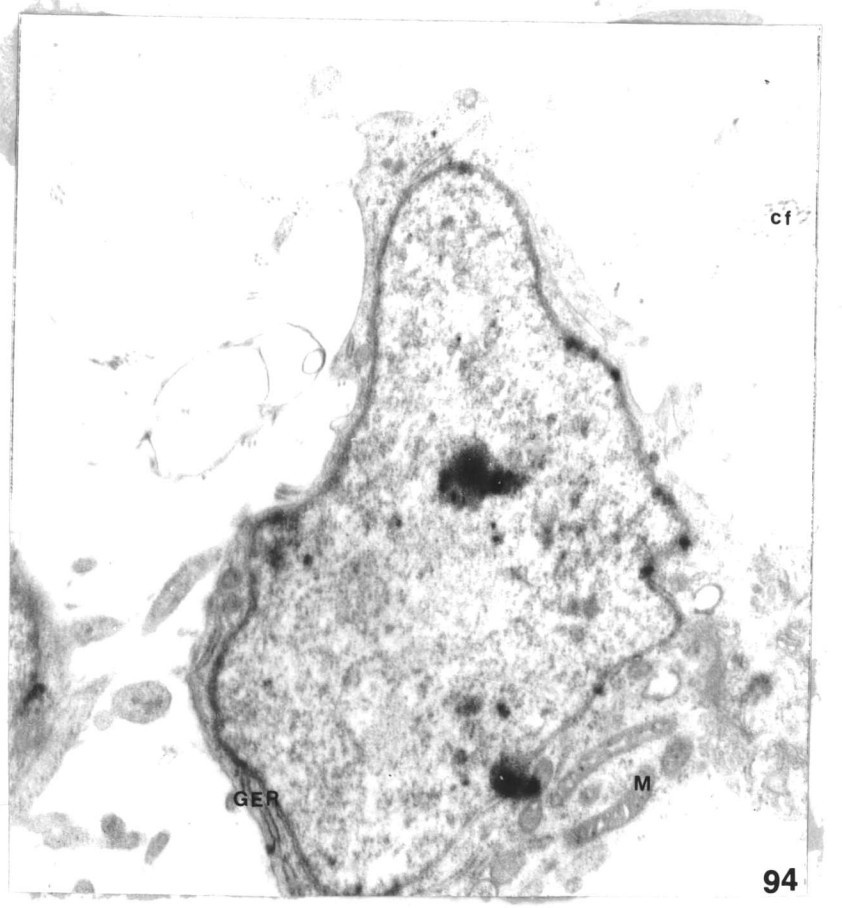
A characteristic of both 9-day control and experimental (Figs. 95, 96,97) was the distinct presence of possible fat droplets or fat inclusions. According to Bloom and Fawcett (1968), some fibroblast cells which at one time secreted fibers may in many instances differentiate into mature fat cells. The 9-day-old control (Fig. 95) revealed an extensive amount of small vesicles, some near the periphery of the cell and others in close association with the Golgi bodies. Granular endoplasmic reticulum was present in the moderate amounts and storage

Fig. 93. Transmission electron micrograph of fibroblasts from skin of 6-day-old saline-treated control embryo. Note the abundance of Golgi (GB), granular ER (GER) and mitochondria (M). Nucleus (NU), nuclear membrane (arrow). X 68,000

Fig. 94. Transmission electron micrograph of fibroblasts from skin of 6-day-old LACA-treated embryo. The lack of Golgi bodies suggests that no secretory proteins are being processed. Mitochondria (M) and granular ER (GER) are present in a scant amount. A few faint collagen fibrils (CF), however, are seen in the extracellular matrix. X 68,000



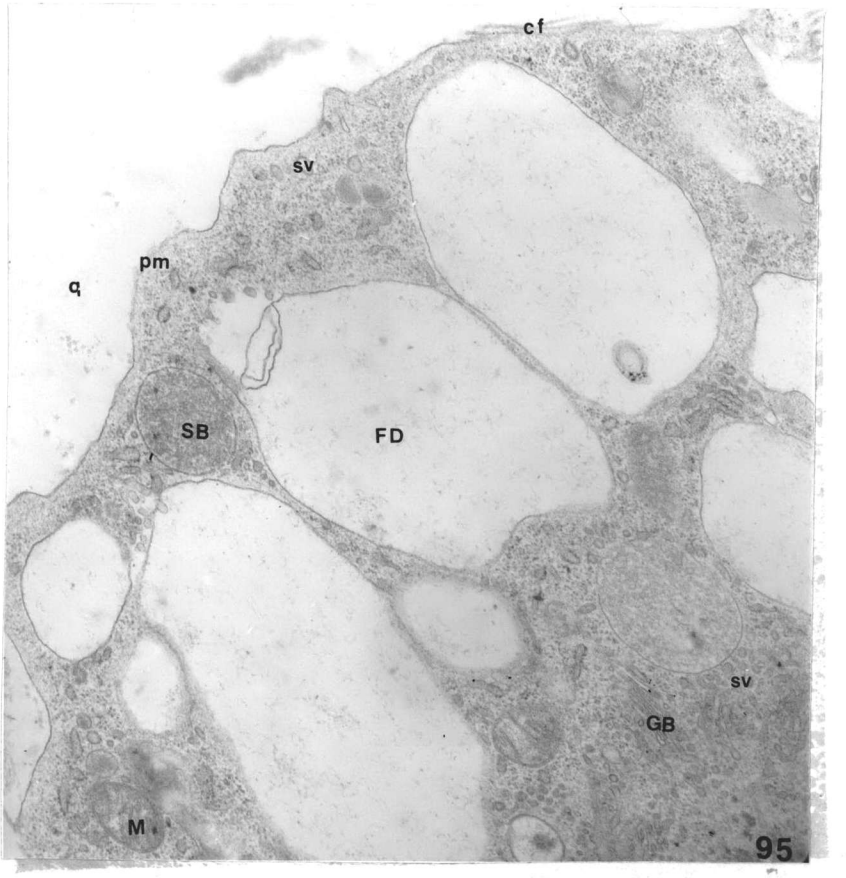
93



94

Fig. 95. Transmission electron micrograph of dermal fibroblast from 9-day-old control embryo undergoing transition into mature fat cell. Note the huge fat droplets (FD) and the storage bodies (SB). Mitochondria (M) are present and Golgi bodies (GB) are distinct. In the extracellular matrix are the deposition of faint collagen fibrils (CF) lying adjacent to the plasma membrane (PM). Small secretion vesicles (SV) are prominent along the periphery of the cell. X 100,000

Fig. 96. Transmission electron micrograph of a similar cell as in Fig. 95, but from the dermis of the 9-day-old LACA-treated embryo. The fat droplers (FD) are present; so are granular ER (GER). Mitochondria and Golgi are not apparent; however, numerous small secretory vesicles (SV) are evident at the plasma membrane (PM). X 68,000


cf
sv
pm
q
SB
FD
sv
GB
M
95

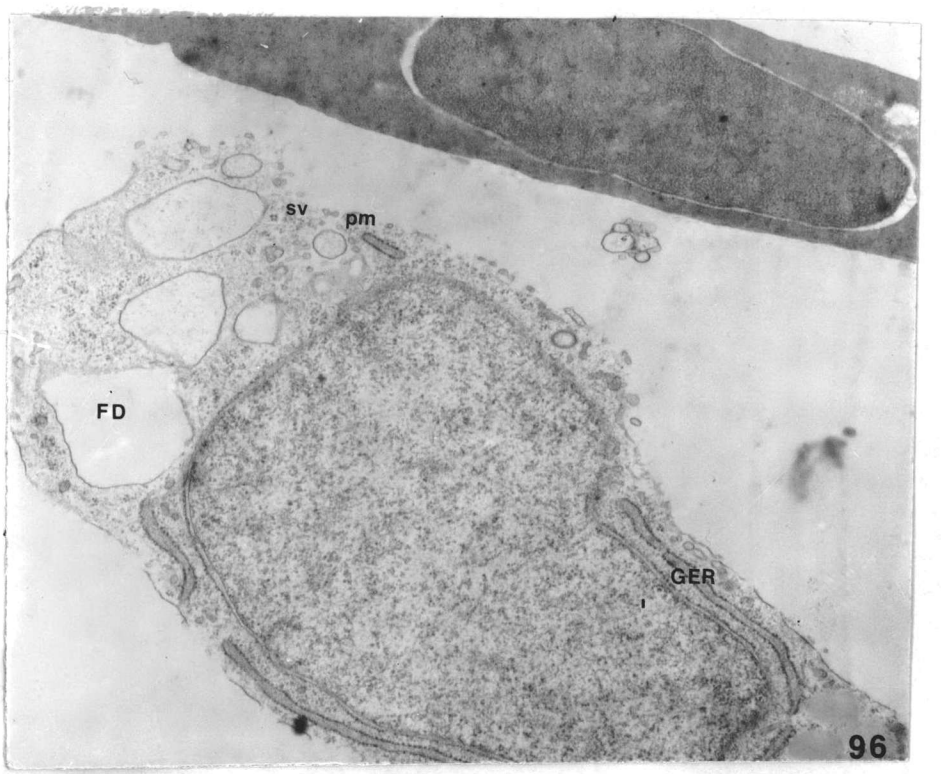
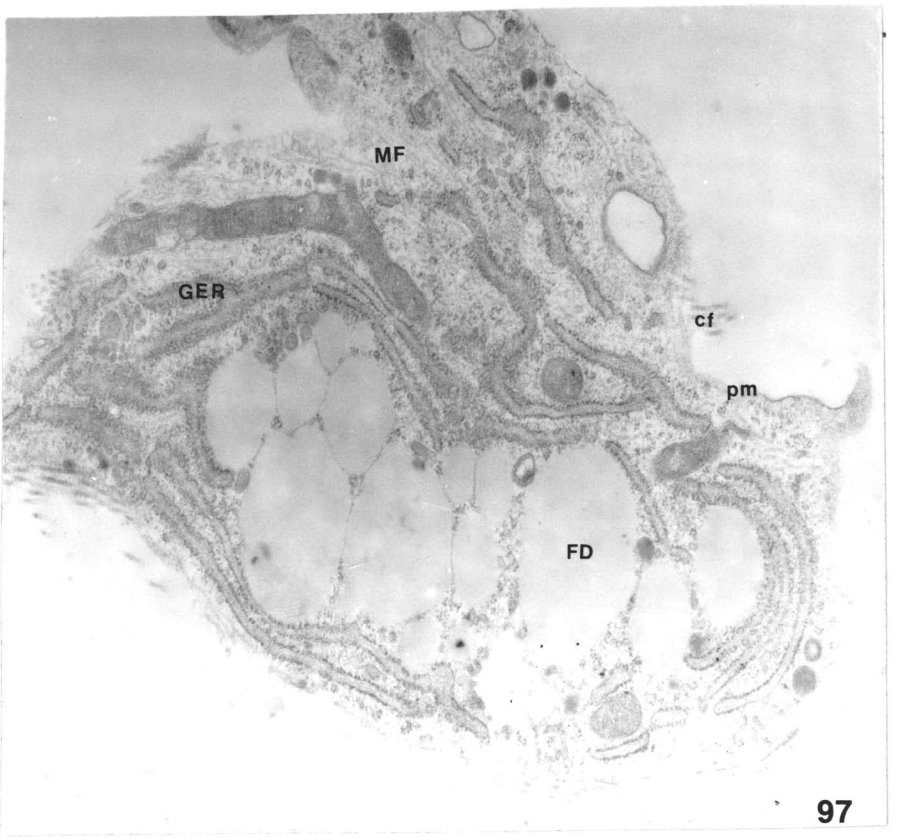
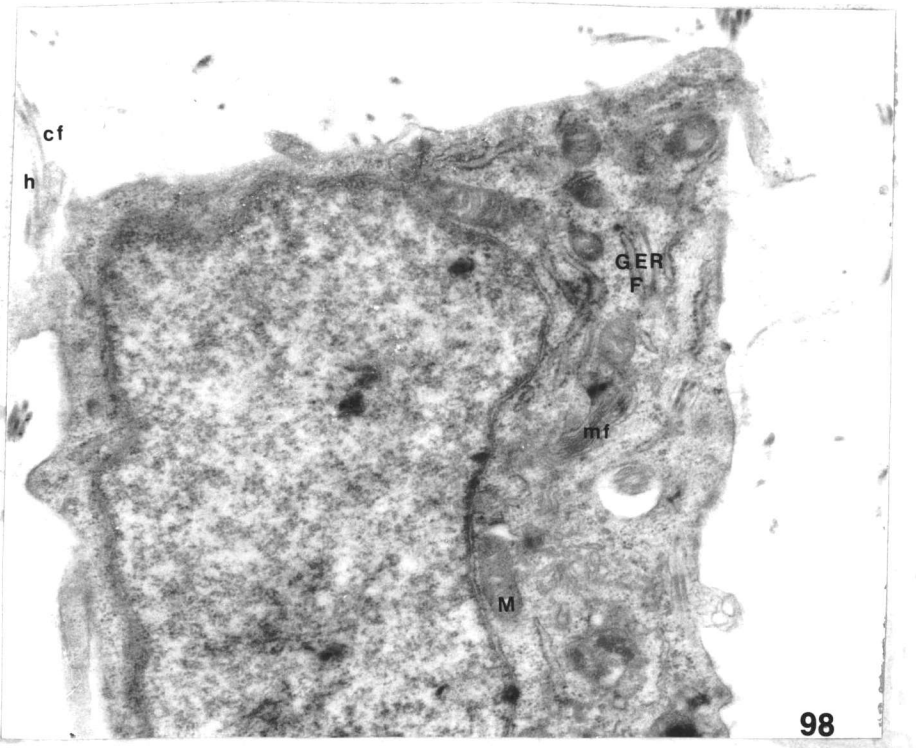

sv
pm
FD
GER
96

Fig. 97. Transmission electron micrograph of dermal fibroblast from 12-day-old saline-treated control embryo. The granular ER (GER) is present in extensive amounts, possibly indicating an increased amount of protein synthesis of secretory molecules - presumably collagen. Collagen fibrils (CF) are seen in close proximity with the external surface of the plasma membrane (PM). Microfilaments (MF) and fat droplets (ED) are also present. X 140,000

Fig. 98. Transmission electron micrograph of dermal fibroblast from 12-day-old LACA-treated embryo. The extensive granular ER (GER) found in the control is not evident in the experimental; however, granular ER is present. Mitochondria (M) are present and so are microfilaments (MF); a scant amount of collagen fibrils (CF) are apparent outside the plasma membrane. X 140,000


MF
GER
cf
pm
FD
97


cf
h
GER
F
mf
M
98

granules were also present in Fig. 95; however, in Fig. 97 (12-day control) the endoplasmic reticulum was present in extensive amounts, indicating an increased amount of protein synthesis, particularly secretory proteins. These membrane-limited elements plus granules conssitute the "ergastoplasm" of these cells (Porter and Pappas, 1959). The profiles have been shown to represent transverse sections through flattened vesicles or cisternae rather than longitudinal sections through tubules. Collagen fibrils were seen in close proximity with the outer surface of the plasma membrane which is non-continuous in these fiber-associated locations. There were also some microfilaments present. The 9-day-old experimentals (Figs. 96,98) contained some small vesicles lying adjacent to the broken membrane, but the presence of collagen fibrils outside the cell was hardly distinguishable. Golgi bodies were detectable and a small to moderate amount of granular endoplasmic reticulum was apparent. The mitochondria were quite distinct (Fig. 96).

By the 12th day, the granular endoplasmic reticulum in the control had increased significantly. Numerous vesicles were seen along the cell's cortex. The collagen fibrils have aligned themselves in a characteristic pattern in the extracellular matrix. These fibrils showed the distinct cross-banding unique to collagen (Fig. 99). The 12-day-old experimental did not exhibit the extensive endoplasmic reticulum seen in the control. Collagen fibrils were present but in a scant amount (Fig. 100). Figures 101 (12-day-old control) and 102 (12-day-old experimental)

Fig. 99. Transmission electron micrograph of dermal fibroblast from 12-day-old saline-treated control embryo. Again note the extensive amount of granular endoplasmic reticulum (GER) that is present in the cell. Storage granules (SG) are visible, but mitochondria and Golgi are not discernible. Microfilaments (MF) are also present. X 140,000

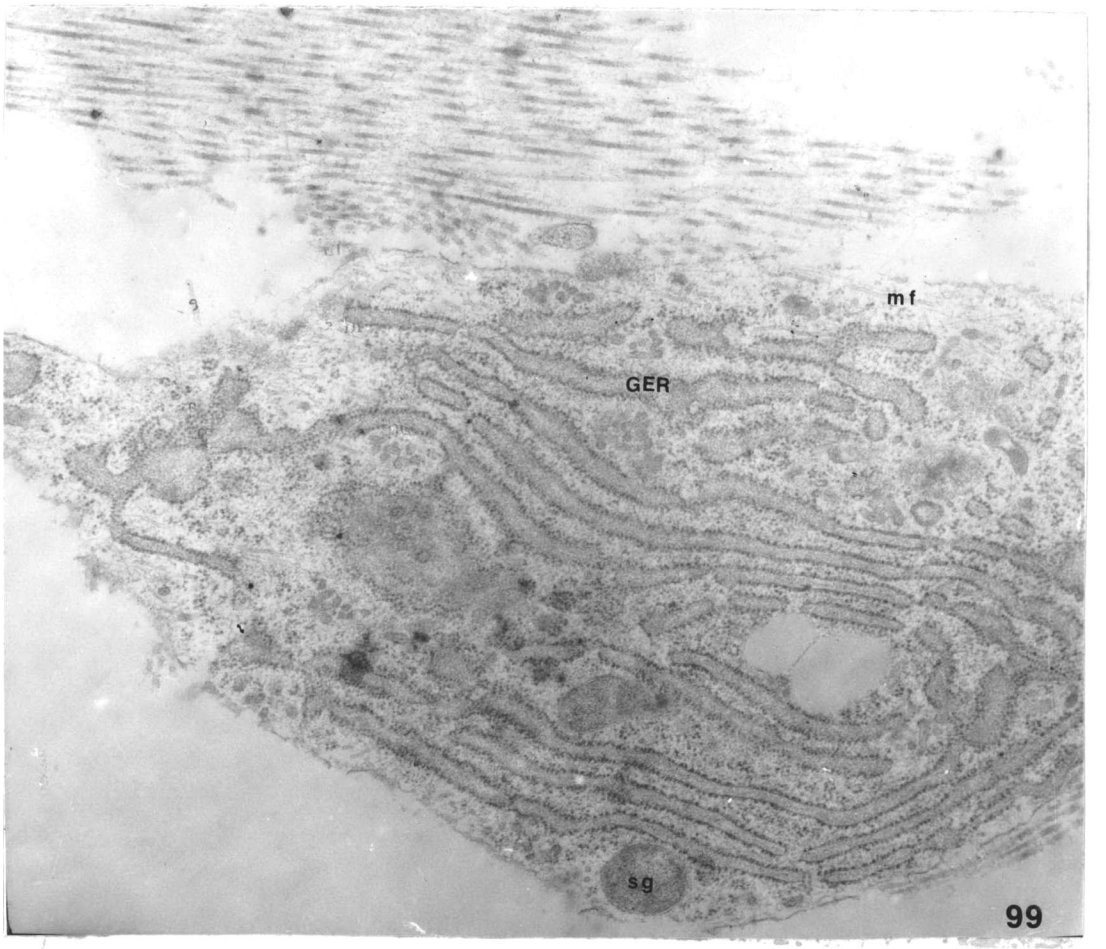

mf
GER
sg
99

Fig. 100. Transmission electron micrograph of dermal fibroblast from 12-day-old LACA-treated embryo. The amount of granular ER (GER) present is not as abundant as that found in the control (Fig. 99). The mitochondria (M) are discernible and so are microfilaments(MF). An interesting feature is the lack of aligned collagen fibrils (CF) adjacent to the plasma membrane. Some fibrils are distinct in the intercellular space but are not as abundant as those found in the control. X 140,000

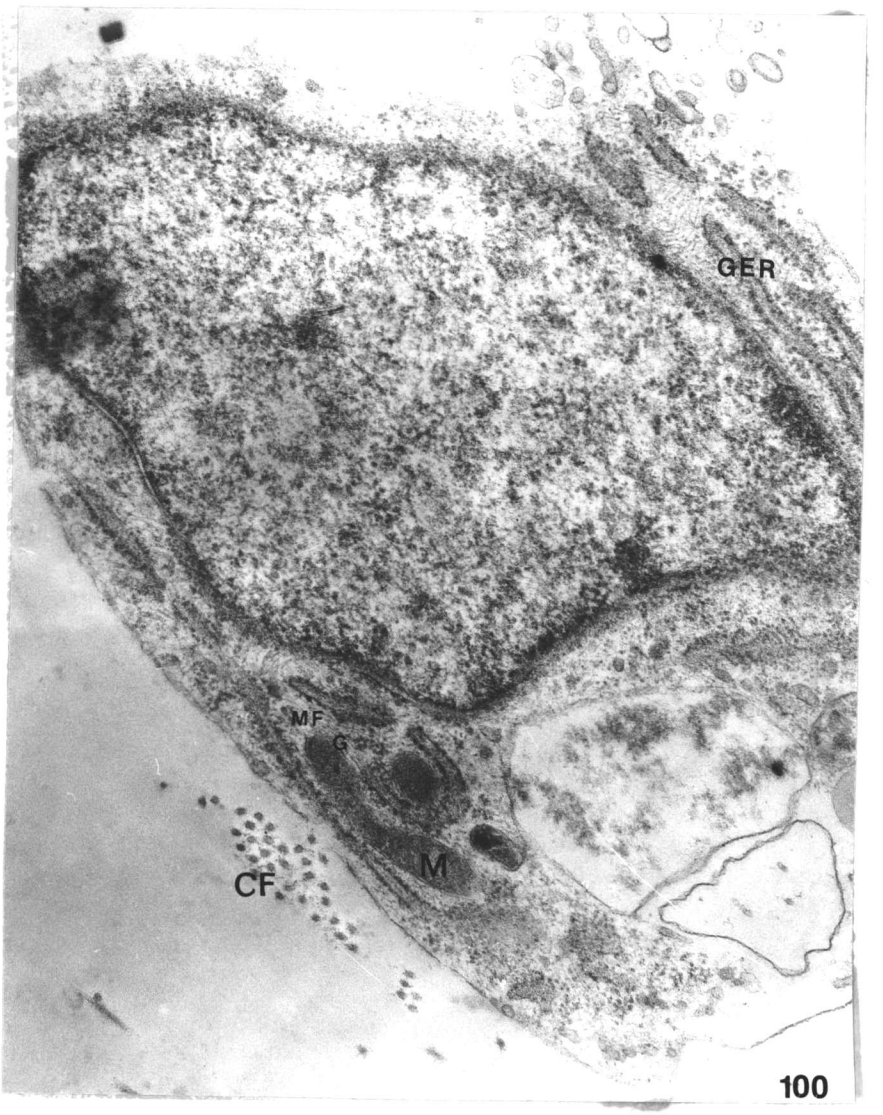

GER
MF
G
M
CF
100

Fig. 101. Transmission electron micrograph of intercellular space of two dermal fibroblasts from the skin of 12-day-old control. Note the alignment of the collagen fibrils (CF) between the adjacent cells. The periodicity of these fibrils is quite distinct (arrows). Filopidia (FP), plasma membrane (PM), granular ER (GER), nucleus (NU), nuclear membrane (NM), and microfilaments (MF). X 200,000

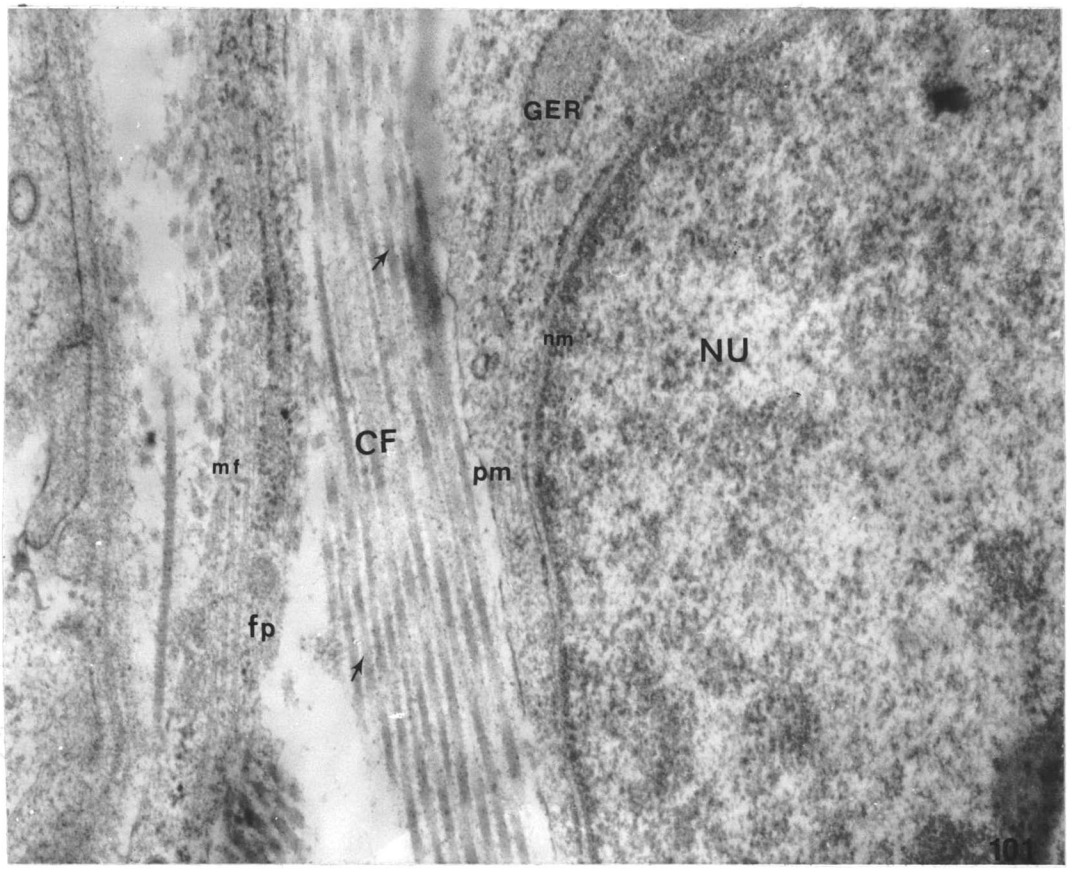
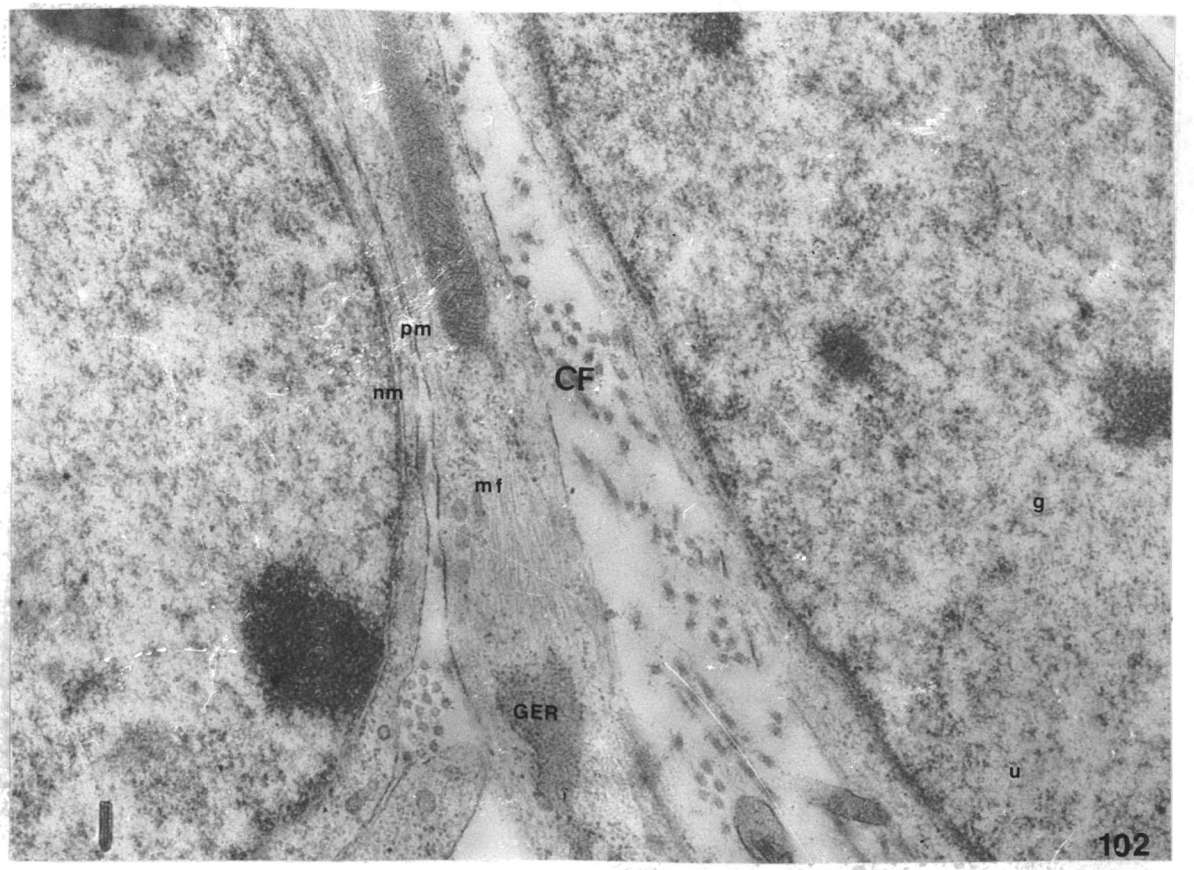

GER
nm
NU
CF
mf
pm
fp
101

Fig. 102. Transmission electron micrograph of intercellular space of two dermal fibroblasts from 12-day-old LACA-treated embryo. Note the lack of aligned collagen fibrils (CF) in the intercellular space; there are some fibrils present, however, but they are much less abundant than those found in the control. Nucleus (NU), plasma membrane (PM), nuclear membrane (NM), filopodia (FP), granular ER (GER), and microfilaments (MF), X 200,000


pm
nm
CF
mf
g
GER
u
102

represent the intercellular space between two adjacent fibroblasts. There was an abundance and compactness of collagen in the control as compared to the sparse and loosely arranged fibrils in the experimental. At this stage during development the fibrils were very prominent; their concentration in the intercellular spaces was much greater than at 9-days (Fig. 103). However, in the 12-day-old experimentals (Fig. 104), fibrils were more prominent than in the 6 and 9-day-old experimentals.

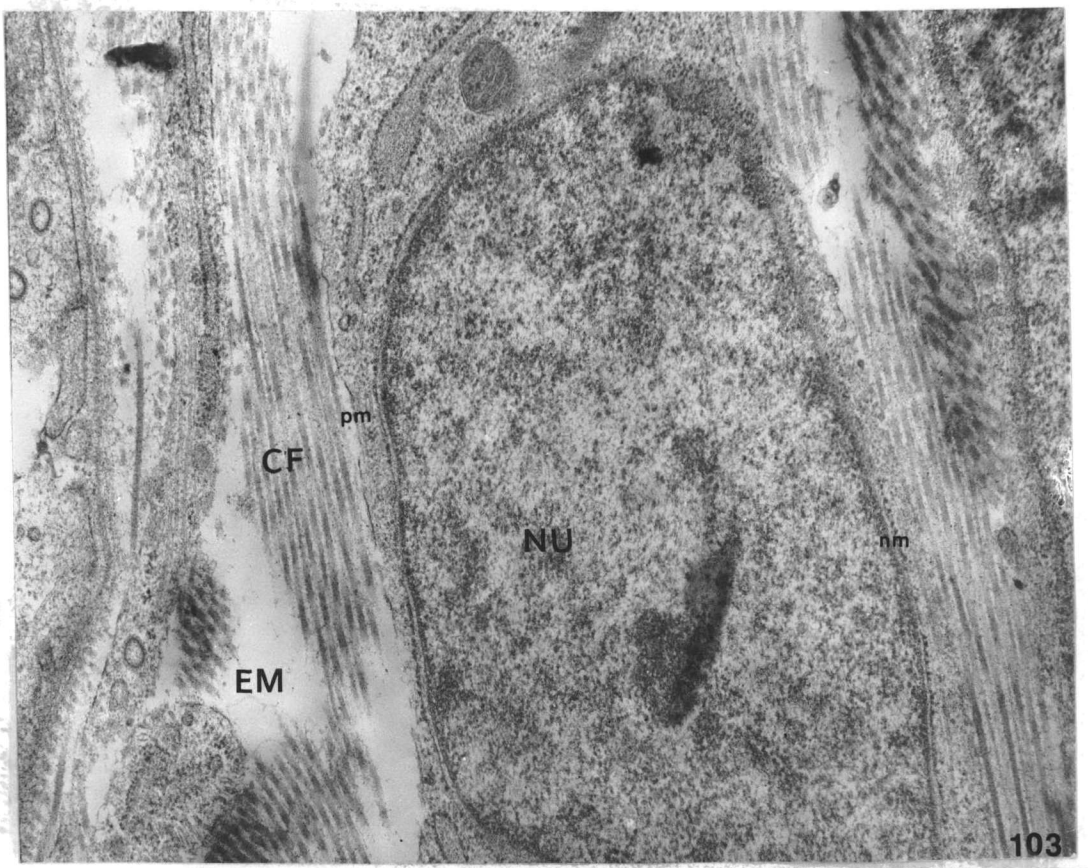
As chick development proceeded to the 14th and 15th day, it became a bit more difficult to distinguish the control fibroblast from the experimental. Figures 105 and 106 represent dermal fibroblasts and filopodia from the control and experimental, respectively. Collagen fibrils were abundant in the matrix. The filopodia of both contained extensive microfilaments. The granular reticulum and mitochondria were in low to moderate amounts. The fibrils of both the control and LACA-treated embryos showed a periodicity that was characteristic of collagen (Figs. 107-108). Fibrogenesis is probably progressing at a much greater rate in the 14-day embryos. By this time, the concentration of intercellular fibrils was much greater than at 12 days (Figs. 107-108). The Golgi bodies were a prominent characteristic of the control cells at this stage (Figs. 109-111). Some experimentals at this stage (14-day-old) did, however, seem to have less concentrated amounts of fibrils in the matrix (Figs. 112-113).

Hydroxyproline Analysis

Table 3 shows that micrograph amounts of hydroxyproline were

Fig. 103. Transmission electron micrograph of dermal fibroblast from 12-day-old saline-treated control embryo showing the compactness of collagen fibrils (CF) in the intercellular space. The fibrils are arranged in a lattice-like fashion. Nucleus (NU), plasma membrane (PM), nuclear membrane (NM), extracellular space (EM). X 140,000

Fig. 104. Transmission electron micrograph of dermal fibroblast from 12-day-old LACA-treated embryo showing sparse amount of collagen (CF). These fibrils are arranged in a lattice-like fashion as seen in the control. Mitochondria (M), granular ER (GER), plasma membrane (PM), nucleus (NU) and nuclear membrane (NM), intercellular space (EM). X 140,000


pm
CF
NU
nm
EM
103

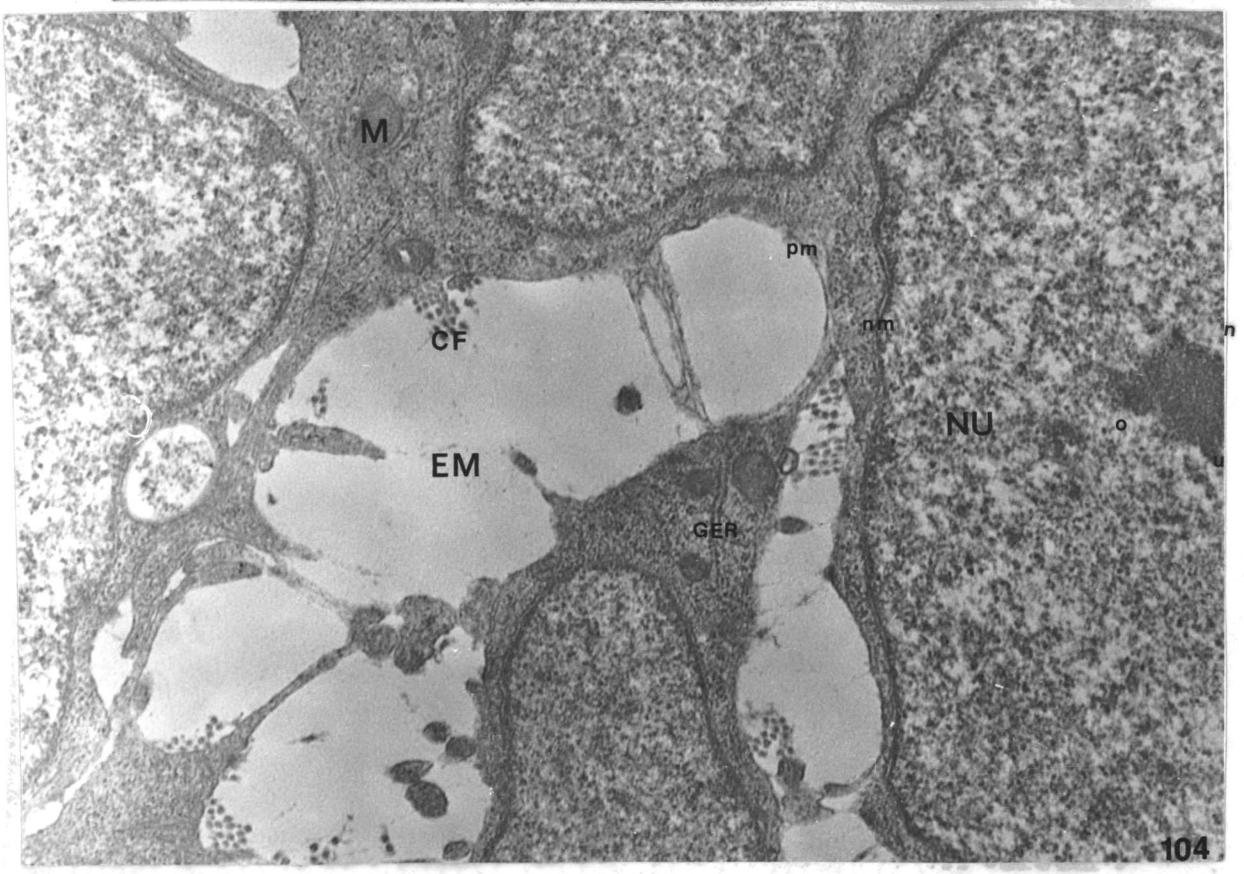
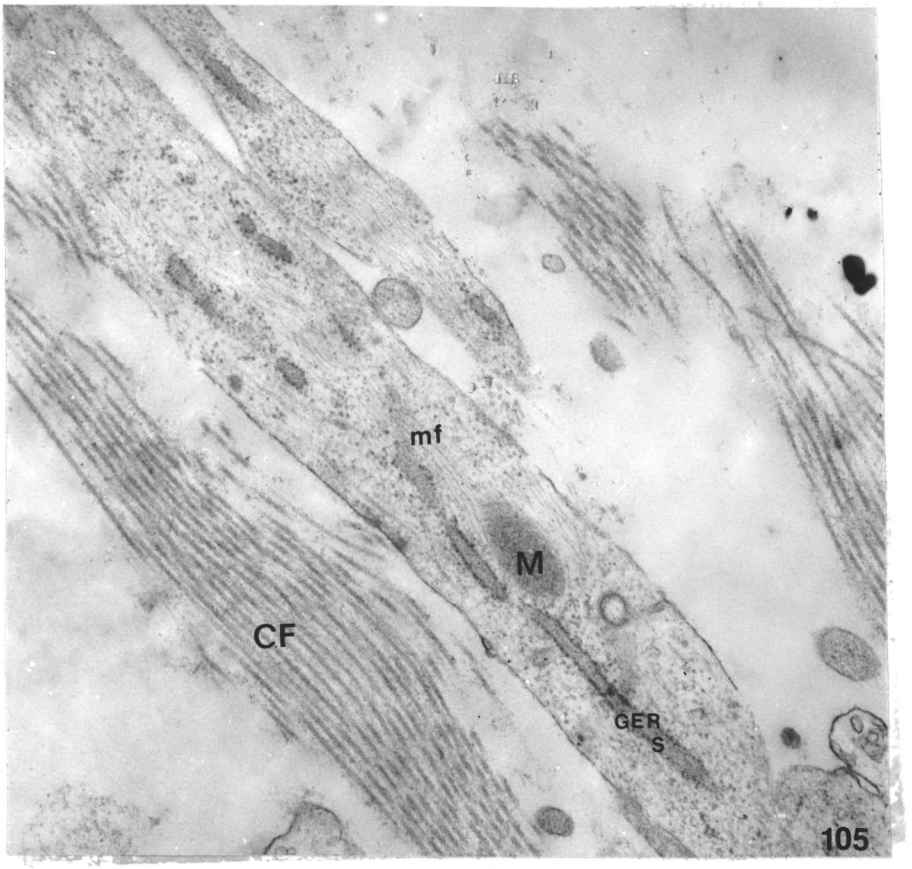

M
pm
nm
CF
EM
NU
GER
104

Fig. 105. Transmission electron micrograph of filopodia from dermal fibroblasts of skin from 14-day-old saline-treated control embryo. Note the abundance of collagen fibrils (CF) in the extracellular matrix. Microfilaments (MF) are present in the filopodia. Mitochondria (M) and granular ER (GER) are also present. X 68,000

Fig. 106. Transmission electron micrograph of filopodia from dermal fibroblasts of skin from 14-day-old LACA-treated embryo. Collagen fibrils (CF) are obvious in the extracellular matrix but the fibrils are not as abundant nor are they as large as those found in the control (Fig. 105). Microfilaments (MF), granular ER (GER), mitochondria (M) as well as nuclei (NU) are evident. X 68,000


mf
M
CF
GER
S
105

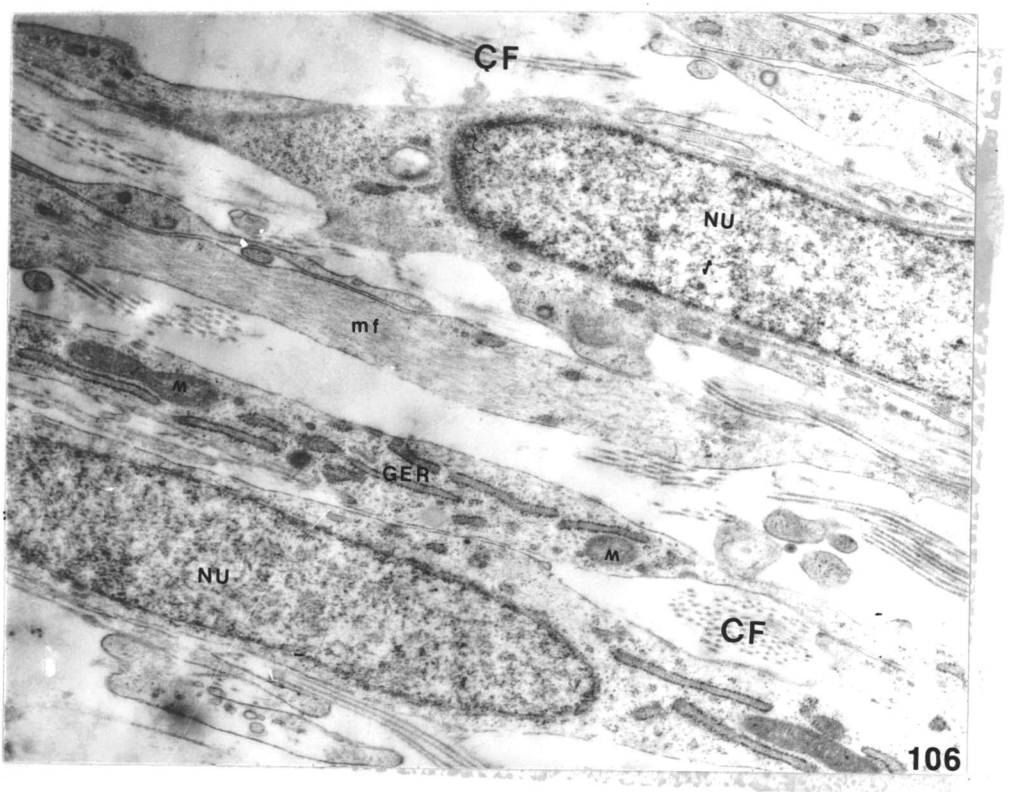
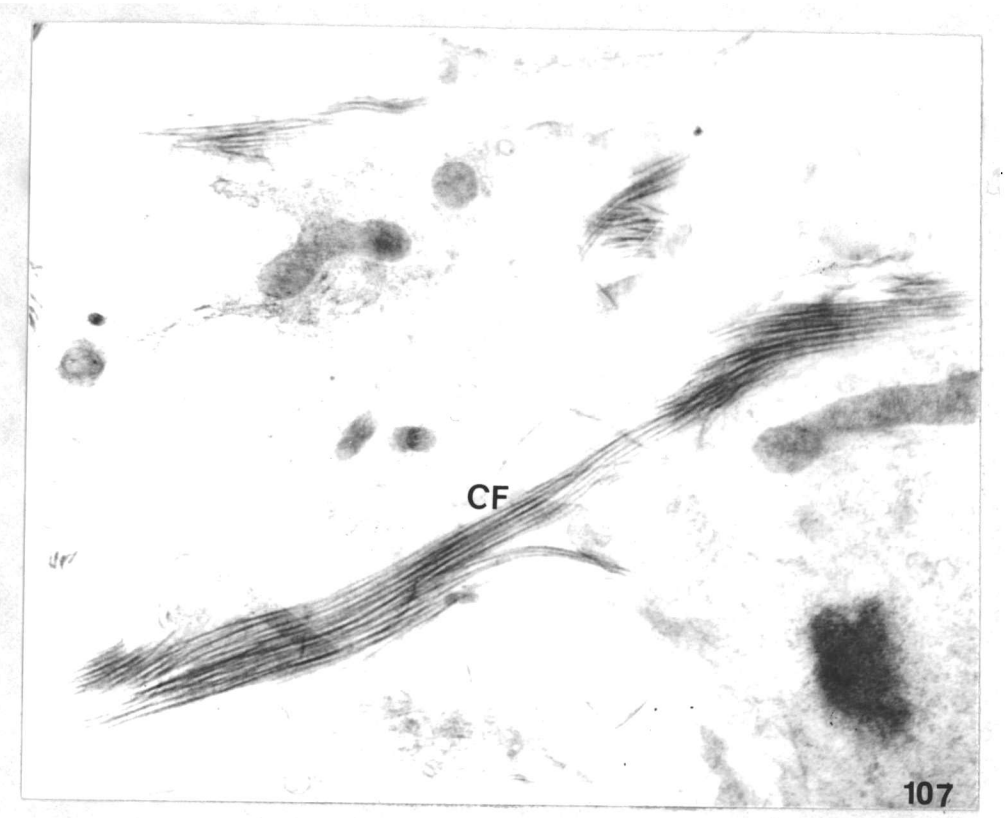

CF
NU
mf
M
GER
M
NU
CF
106

Fig. 107. Transmission electron micrograph of collagen fibrils secreted by dermal fibroblasts of the skin of 14-day-old saline-treated control embryo. The collagen fibrils (CF) have a slightly beaded appearance showing a distinct periodicity of the fibrils. X 100,000

Fig. 108. Transmission electron micrograph of collagen fibrils adjacent to dermal fibroblasts of skin of 14-day-old LACA-treated embryo. The fibrils (CF) do not differ from those of the control; they are both aligned in a similar pattern and the periodicity is prominent. X 200,000


CF
107

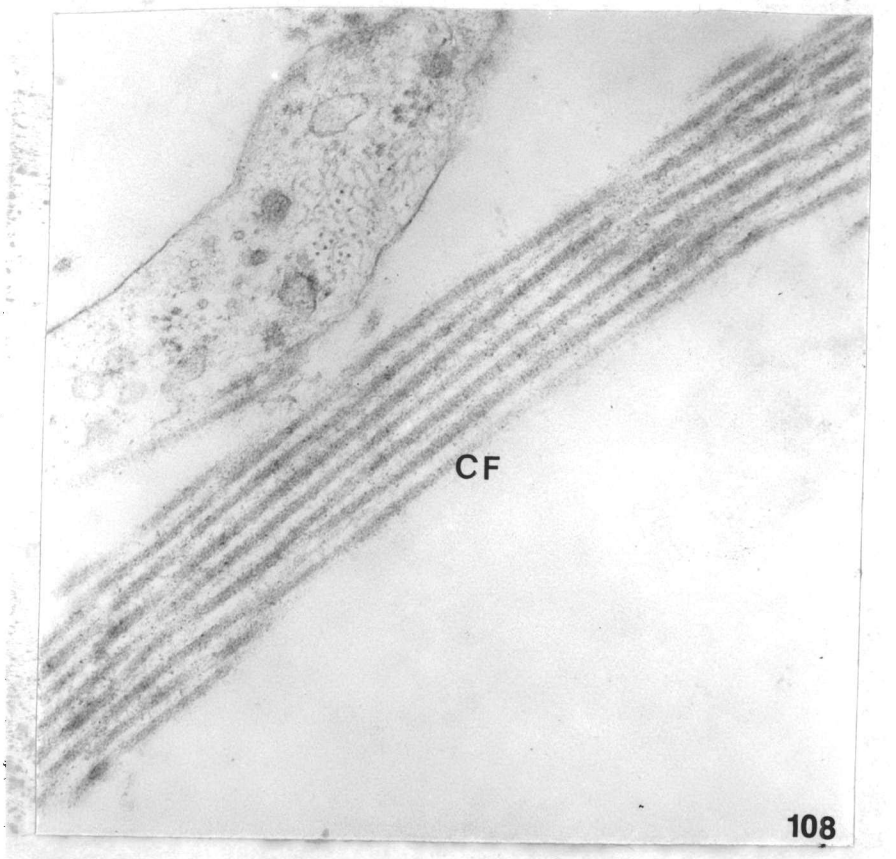

CF
108

Fig. 109. Transmission electron micrograph of filopodia of dermal fibroblasts from 14-day-old saline-treated control embryo. Note the abundance of collagen fibrils (CF) in the extracellular matrix (EM). The fibrils exhibit a distinct alignment and their characteristic periodicity (arrows). The Golgi bodies (GB) are prominent, as are the granular ER (GER) and mitochondria (M). The microfilaments (MF) are also visible. X 68,000


m1
CF
GER
EM
M
GB
CF
109

Fig. 110. Transmission electron micrograph: filopodia of dermal fibroblast from 14-day-old saline-treated control embryo. Again, note the numerous bundles of collagen fibrils (CF) aligned in the extracellular matrix (EM). The periodicity of the fibrils is distinct. Golgi bodies (GB), mitochondria (M), granular ER (GER) and microfilaments (MF) are apparent. X 68,000


mf
GB
CF
GER
EM
M
110

Fig. 111. Transmission electron micrograph of dermal cell from 14-day-old saline-treated control. The Golgi bodies (GB) in the cell are very prominent and so are secretion vesicles (SV). Microfilaments (MF) are evident and so are huge storage bodies (SB).

X 100,000

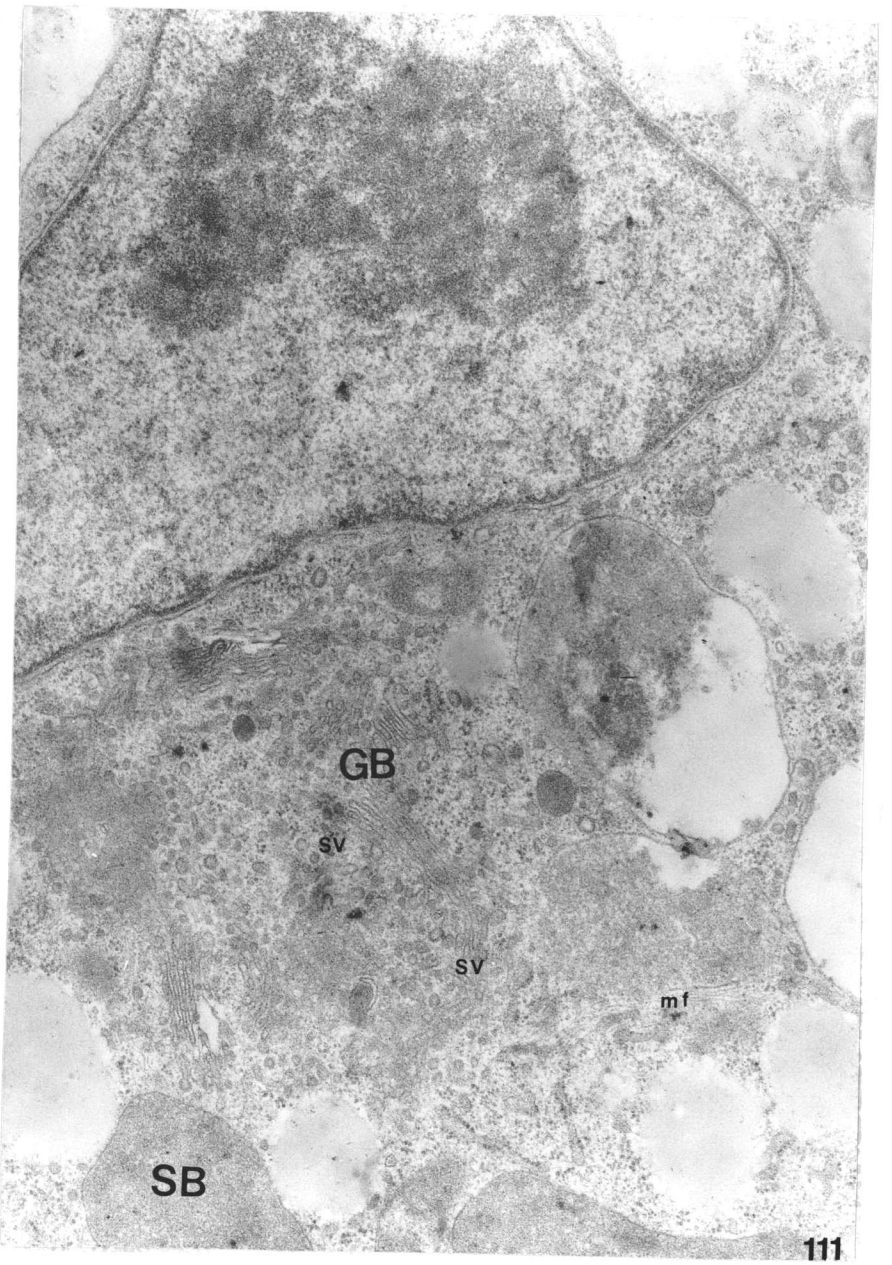
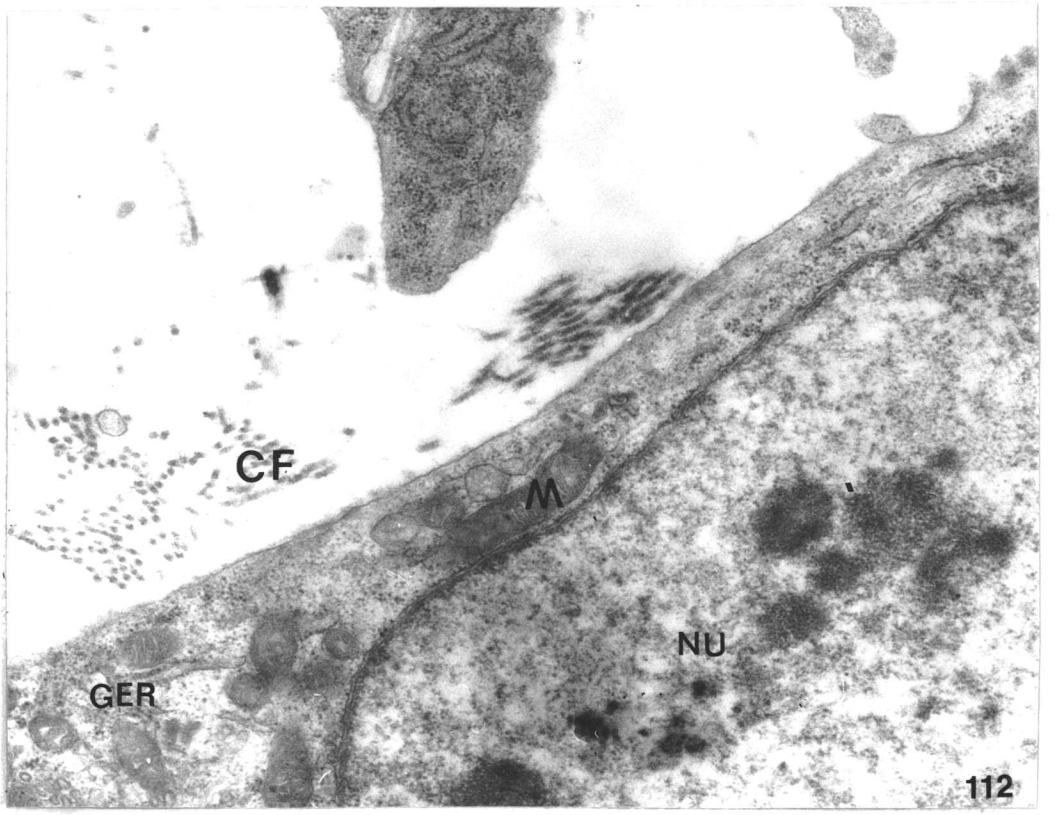

GB
SV
SV
mf
SB
111

Fig. 112. Transmission electron micrograph of dermal fibroblast from skin of 14-day-old LACA-treated embryo. Note the amount and unorganized arrangement of the collagen fibrils (CF) found adjacent to the plasma membrane. Mitochondria (M) granular ER (GER), nucleus (NU), nuclear membrane (NM). X 48,000

Fig. 113. Transmission electron micrograph of dermal fibroblasts from skin of 14-day-old LACA-treated embryo. Note the initial assembly of collagen fibrils (CF) just outside the plasma membrane (PM). These fibrils too seem to be more loosely arranged than those fibrils of the control at the same age. X 48,000


CF
M
NU
GER
112

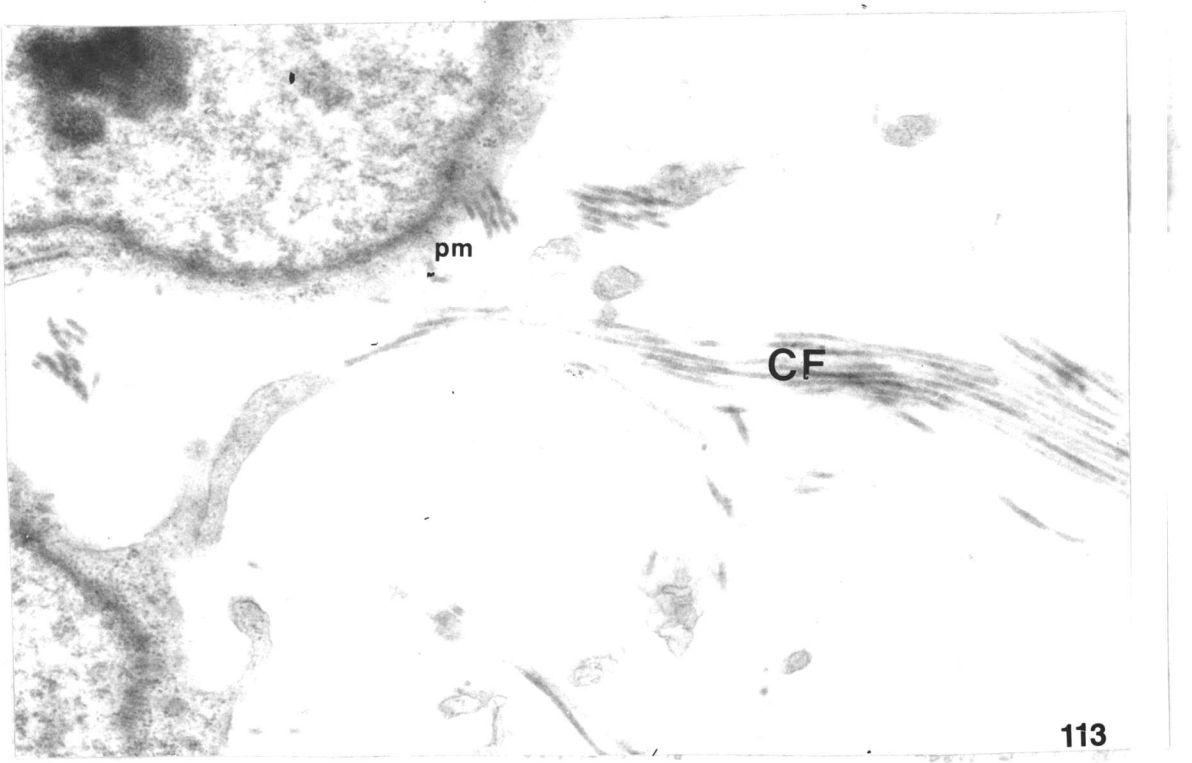

pm
CF
113

Table 3. Analysis of Hydroxyproline (μg/gm skin) in control and LACA-treated embryos.

	Age of Embryos (Days)				
	Group I	Group II	Group III	Group IV	Group V
	6-8	9-10	11-12	13-14	15-16
CONTROLS					
0.9 cc Saline	.390	1.47	5.10	6.70	9.75
0.6 cc Saline	.388	1.48	5.00	6.72	9.77
EXPERIMENTALS					
* 0.9 cc LACA	.255	1.11	3.05	6.00	9.40
**0.6 cc LACA	.090	.450	1.23	3.95	8.38

* Single injections of LACA
**Triple injections of LACA (0.6 cc/injection)

determined per gram (wet weight) of dorsal skin in chicken embryos. A comparative study was performed between control and experimental embryos receiving only single injections (0.9 cc of saline and 0.9 cc of LACA, respectively) and from those receiving triple injections (0.6 cc of saline and 0.6 cc of LACA/injection). The microgram amounts of hydroxyproline found in both types of controls did not differ significantly. The highest difference was approximately a tenth of a microgram (Table 3, Group III, 11-12 day). However, the experimentals receiving a total of 1.8 cc of LACA had a much lower level of this amino acid per gram wet weight when compared to those embryos that received a total of 0.9 cc of LACA. Additionally, both experimentals had lower amounts of hydroxyproline than the corresponding controls (Table 3). As the amounts (cc) of LACA increased from 0.9 to 1.8 within the chick embryo's environment, the level of hydroxyproline, as measured by a modification of Neuman and Logan (1950), decreased for each group examined. The amounts (μg) of hydroxyproline determined in both control and experimental embryos increased steadily as development and skin morphogenesis progressed. LACA was administered to the embryos before the third day of incubation; it did not seem to take its greatest effect on hydroxyproline concentration between 15-16 days of development and it appeared to have an even lesser effect on hydroxyproline concentration in embryos between 6-8 days of development. The smallest decrease in the hydroxyproline concentration (when compared to the controls)

occurred on or around these previously mentioned days (Table 4, Figs. 114-115). The largest decrease in the amount of hydroxyproline due to LACA-treatment, occurred on or around days 11-12.

Table 4. Decrease in the amount of hydroxyproline (µg/gm skin) found in LACA-treated embryos as compared to controls.

Embryo's Age (days)	Single Injection (saline)	µg of *HYPR	Single Injection (LACA)	µg of HYPR	Triple Injection (saline)	µg of HYPR	Triple Injection (LACA)	µg of HYPR
6-8	0.9	.390	0.9	-.135	0.6	.388	0.6	-.298
9-10	0.9	1.47	0.9	-.360	0.6	1.48	0.6	-1.03
11-12	0.9	5.10	0.9	-2.05	0.6	5.00	0.6	-3.77
13-14	0.9	6.70	0.9	-.700	0.6	6.72	0.6	-2.77
15-16	0.9	9.75	0.9	-.350	0.6	9.77	0.6	-1.39

*Hydroxyproline

Fig. 114. Standard curve for hydroxyproline (µg amounts).

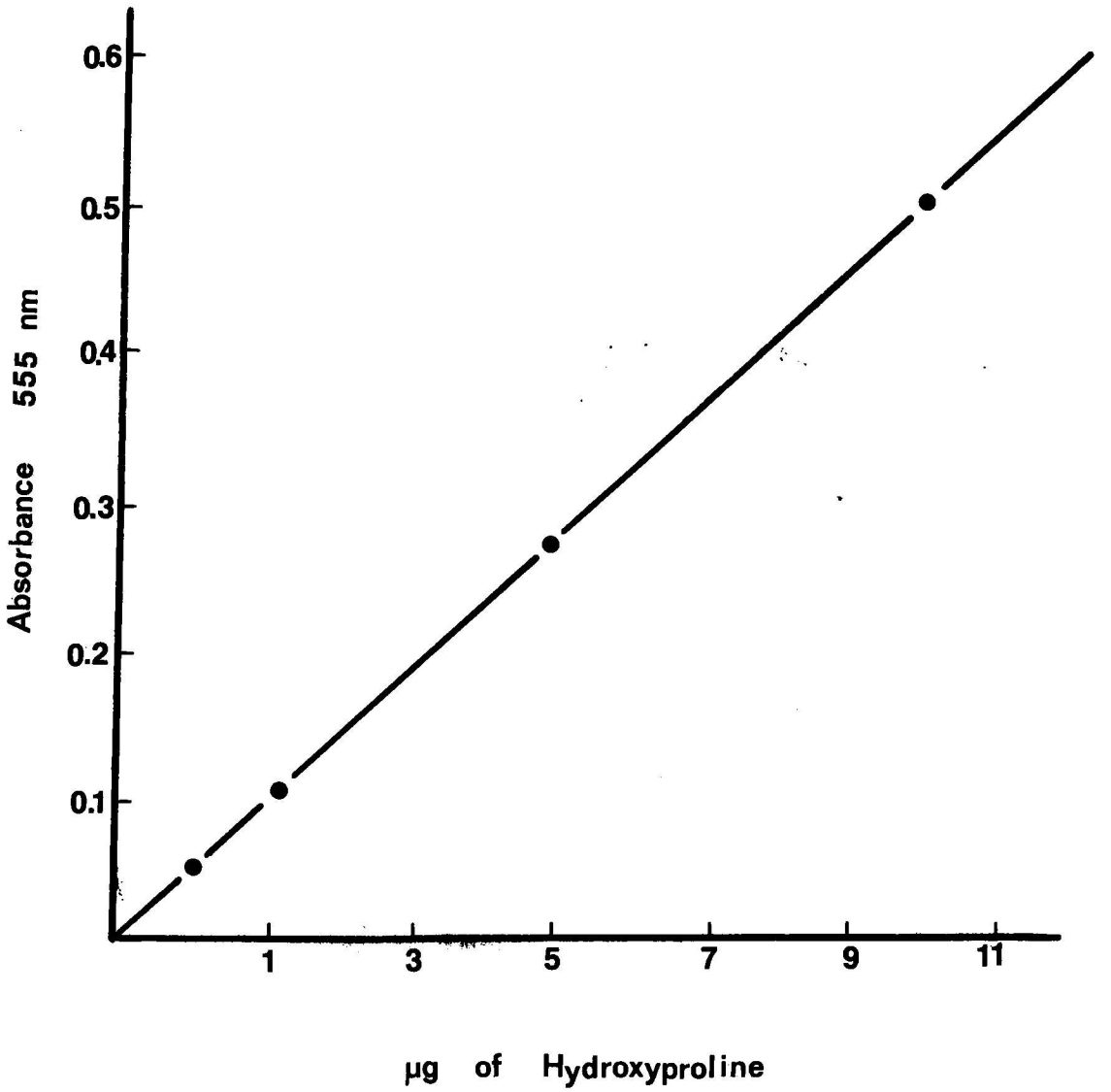
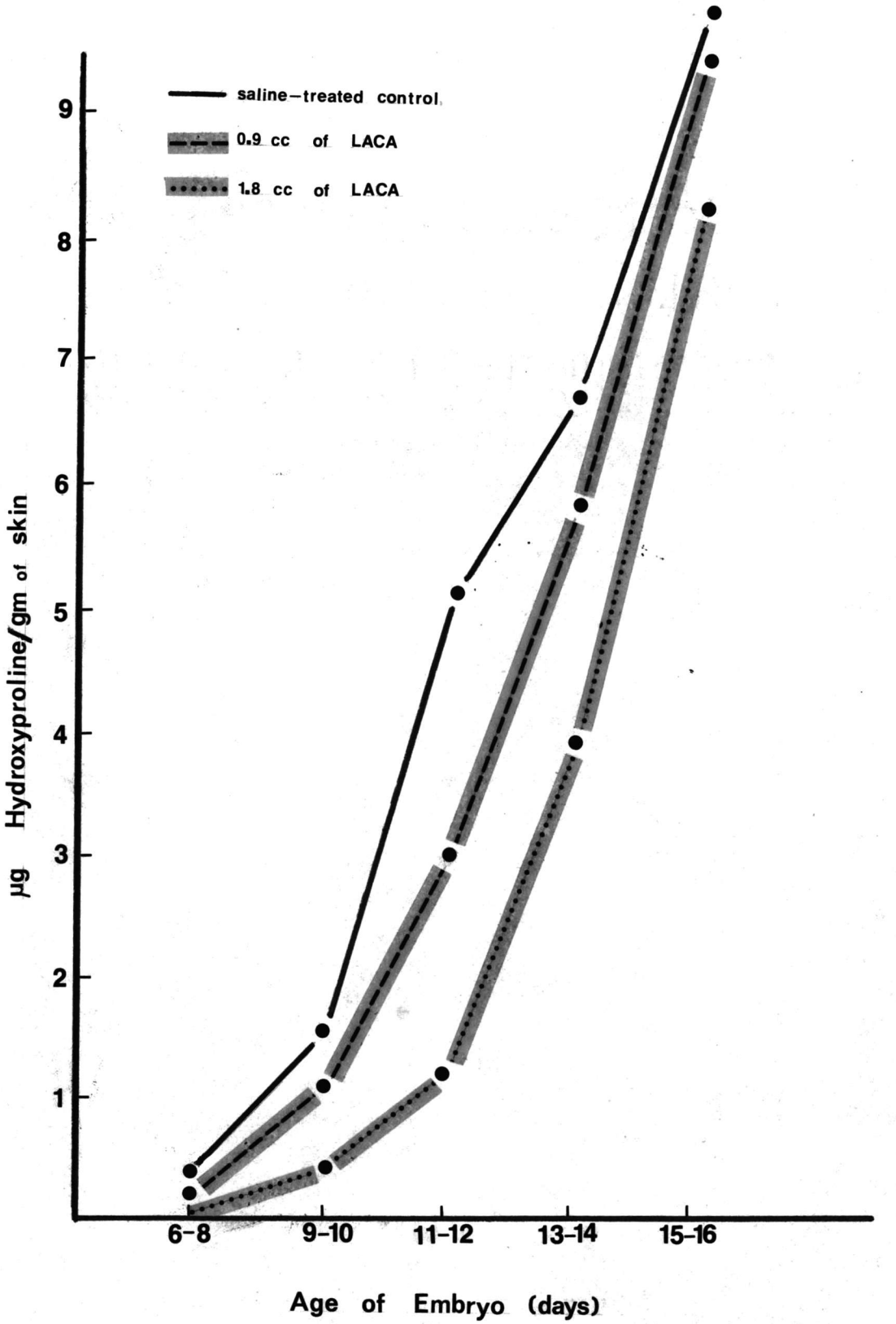

0.6
0.5
0.4
0.3
0.2
0.1
Absorbance 555 nm
1
3
5
7
9
11
µg of Hydroxyproline

Fig. 115. This graph represents the μg amounts of hydroxy-proline found per gram wet weight of skin in chicken embryos.

Saline-treated control

LACA-treated experimental (Single injections)

LACA-treated experimentals (Triple injections)


saline–treated control
0.9 cc of LACA
1.8 cc of LACA
µg Hydroxyproline/gm of skin
1
2
3
4
5
6
7
8
9
6–8
9–10
11–12
13–14
15–16
Age of Embryo (days)

CHAPTER V

DISCUSSION

Macroscopic

The age at which injections of L-azetidine-2-carboxylic acid were made and the amount given at that particular time were critical. When embryos were given single injections of 0.9 cc of LACA at 16-18 hr, more feather suppressions occurred at this time alone than from single treatments at pre-incubation, 24, and 48 hr. At pre-incubation 0.9 cc of LACA was apparently toxic to the embryos. When embryos were given triple injections, a total of 1.8 cc of LACA (0.6 cc/injection), more feather suppressions occurred in these embryos than in embryos receiving single injections at 16-18 hr. After the injections were made, the embryos were analyzed grossly for feather suppressions at various days during development. When observed at day 12, more embryos exhibited feather suppressions than at any other age. The general conclusion drawn from these data was that 16-18 hr injection time and 11-12 days of incubation evidently represented critical periods for LACA-inhibited events with respect to embryonic growth, and that the administration and results of LACA, respectively, at such times had a more pronounced effect on embryonic growth than at earlier or later times. More feather suppressions occurred from treatment with 1.8 cc of LACA than 0.9 cc probably because the increased amounts of LACA caused more of the X and Y positions, of the collagen molecule that

are normally occupied by proline and hydroxyproline, to be occupied by LACA (Prockop et al., 1979).

Why the embryonic chick skin should be so much more sensitive to treatment with LACA at certain hours or certain ages of incubation than at other times is not known, but some aspects of sensitive periods during embryonic development have been discussed by Hall (1977). Diegelman and Peterkofsky (1972) have provided data on collagen biosynthesis over 5 to 12 days of incubation of chick embryos when the rate of collagen synthesis increases some 800%.

LACA acts, in part, by reducing the rate of collagen synthesis (Lane et al., 1971) and by so doing, could slow embryonic growth. The presence of LACA could also cause a reduction in the concentration of extracellular collagen, thus providing a basis for the abnormal skin morphogenesis. Lane et al. (1971) stated that most of the physical properties of the collagen from 12-day-old analog-treated embryos were the same as normal collagen and, therefore, it may be unlikely that the increased number of feather suppressions is the result of any unusual property of the collagen molecule containing LACA.

Skin Whole Mounts

Treatments of triple injections of LACA into chick embryos caused an alteration in the distribution of papillae along the mid-dorsal region of the skin. Cairns and Saunders (1954) and Rawles (1963) have performed extensive studies on inductive interactions between dermis and epidermis during the development of feather germs in the chick embryo. However, very little is known about the morphogenetic mechanisms which determine the precise area of feather germ induction and their characteristic patterns of distribution. Stuart and Moscona

(1967) found that in feather forming areas of the dorsal skin a portion of the dermal cell population becomes organized into distinctly oriented tracts of elongated cells; this process coincides with the deposition of tracts of fibrous material which closely corresponds in location to the tracts of the dermal cells. This of course suggests that feather morphogenesis in the feather-forming areas is closely associated with the formation of fibrous tracts of collagen and that the latter may provide an integrating framework for the distribution pattern of developing feathers in the dorsal skin. The relationship of this fibrous lattice of collagen to skin morphogenesis is suggested by observations on embryos treated with hydrocortisone in which feather development is either totally or partially inhibited (Ede et al., 1971). In cases of total inhibition, neither oriented dermal cell tracts nor an organized fibrous framework was found. In cases of partial inhibition, fibrous strands and oriented dermal cells were found only in regions containing dermal papillae.

In view of previously cited observations, along with those presented here, it is logical to assume again that LACA is either completely inhibiting or decreasing the rate at which collagen fibers are deposited in these specific tracts that correspond in location to the tracts of the dermal cells. That is, the integrating framework does not form at all or does not form at the precise time that the dermal cells become organized into distinctly oriented tracts of elongated cells, thereby causing a suppression or total inhibition of the normal distribution

and growth of dorsal papillae. Essentially, the organization of collagenous fibers in the dermis into distinct areas and arrangements of grouped fibrils precedes the manifestation of the feather buds and as a result dictate embryonic dermal morphogenesis (Stuart and Moscona, 1967). This suggests that the specific arrangements of these collagen fibrils function in the alignment of dermal cells in early embryonic integument and thus organizing the ordered pattern of the developing feather tracts.

Light Histology

Light histology has provided information concerning the morphogenetic changes occurring in the epidermis and dermis of the normal and LACA-treated embryo during feather development. There were several distinct differences or abnormalities that were apparent in the dermis and epidermis of the LACA-treated embryos such as (1) suppressed outgrowth and elongation, (2) decrease in the density of dermal condensations and lack of dermal condensations, (3) lack of basement membrane formation at the epidermal—dermal junction, (4) inhibition of epidermal placodes and unorganized epidermal placodes, and (5) suppression of barb-vane ridge formation.

The possible action of LACA, as a proline analog, on collagen dependent systems such as the integument is known. But how is it that this action of LACA on the collagen molecule is able to interfere with the normal development of the skin; that is, how do abnormal morphogenetic changes in the dermis, presumably due to some alterations either in the structure, synthesis, or extrusion of the collagen

molecule, cause abnormal morphogenetic changes in the epidermis and vice versa? Or simply, what is the relationship between the dermis and epidermis in feather development?

The feather is an expansion of the feather germ; it is composed of an epidermal sheath and a dermal core. Between the time the dorsal ectoderm closes over the neural tube and the first feather germs appear, the dorsal integument acquires the information necessary for its subsequent development. The axial organs (neural tube, notochord, myosclerotome) seem to cause the formation of the feather germs of the spinal pteryla (Sengel, 1964). He states that the early removal of a piece of the neural tube and notochord prevents the differentiation of a portion of the spinal pteryla. The longer the excised piece, the greater the number of suppressed or inhibited feather germs. Inversely, the insertion of a piece of neural tube, notochord or myosclerotome under the skin of a normally glabrous region causes the differentiation of an additional feather field. So, the axial organs seem to have an instructive or decisive role in the organization of the spinal pteryla. It should be made clear that all of the axial organs mentioned previously are composed of connective tissue. This means that just like the integument, they are composed of collagen fibrils, which suggests that their normal morphogenesis and development is affected by the exposure of the embryos to LACA. Therefore, these abnormal axial organs could be another factor in causing the abnormal differentiation and development of the spinal pteryla and this along with the action of the analog on

collagen exert a dual adverse effect on the development of the integument.

So the mechanisms of outgrowth of the feather germ have been precisely elucidated. Each of the small masses of cells induced by the axial system is formed in the dermis during the 6th or 7th day of incubation. The dermis then induces the overlying epidermis to form a feather bud. This activity is followed by an opposite induction coming from the epidermis, acting on the dermis. From then on the epidermis has the principal role in the growth of the feather germ.

In conclusion, the feather germ is the result of numerous reciprocal inductions during which the dermis and epidermis play, one after the other, the role of inducing and reactivating tissues. A striking example of the importance of each of these morphogenetic actions is found in this light histological study of the LACA-treated embryo. LACA affected the differentiation of the embryonic integument directly through its action on the collagen molecules secreted by the dermal fibroblast and indirectly by interfering with the normal differentiation of the axial system which subsequently prevents normal inductive interactions between the axial organs and the dermis.

Ultrastructure

The scanning and transmission analyses of the mid-dorsal region of the skin served to illustrate the ultrastructural changes during the differentiation process *in ovo*. The scanning analysis showed four distinct abnormalities in the integument of embryos treated with LACA. They were; (1) the obvious suppression and/or inhibition of normal feather development, (2) the lack of distinct extracellular fibrils in

the dermis, (3) unorganized arrangement of dermal tissue, and (4) "epidermal blistering" of the peridermal sheath in apterylar regions. The transmission analysis also showed some distinct differences in the skin of LACA-treated embryos when compared to the control, such as (1) decrease in the number of fibrils at the epidermal-dermal junction, (2) noticeable decrease in the number of fibrils associated with the dermal fibroblasts, (3) unorganized lattice arrangement of the collagen fibrils, and (4) some distinct differences in the cytoplasmic integrity. The abnormalities observed with the transmission analysis are ultimately expressed as gross features in the scanning analysis.

The results here demonstrate that L-azetidine-2-carboxylic acid affects the collagen accumulation in chick embryos and thus aberrant morphological changes in the skin and other connective tissues are observable. Since Lane et al. (1971) have shown that the physical properties of the collagen from 12-day-old LACA-treated embryos were the same as normal collage, the assumption here is that the above abnormalities may be due to the decrease in collagen accumulation in the extracellular matrix. This decrease in accumulation could be explained either by a decrease in the rate of collagen synthesis or an increase in the rate of collagen degradation. Lane's data indicated that the major effect is a decrease in the rate of synthesis. Experiments with cartilage in vitro (Takeuchi et al., 1969) demonstrated that the principal effect of specific proline analogs in the first 2 hr or so is to increase the intracellular pool of collagen because the collagen containing the analog cannot be extruded at a normal rate. Autoradiographs from embryos

labeled *in vivo* (Diegelmann and Peterkofsky, 1972) indicated that continuous treatment of LACA to cells for 5 days does not allow for adaptation of these cells to repeated exposure to the proline analog. They still do not extrude collagen containing the analog at a normal rate. So, one would think that these cells are probably in a new steady state in which the decrease in the rate of extrusion is balanced by a decrease in the rate of synthesis or an increase in the rate of intracellular degradation. Experiments in which the embryos were labeled *in vivo* with ^{14}C proline indicated that the rate of collagen synthesis was decreased. Even after relatively short labeling periods there was a decrease in the amount of ^{14}C incorporated into collagen relative to that incorporated into other proteins (Takeuchi and Prockop, 1969). The same experiment indicated that there was no marked increase in the rate of extracellular or intracellular degradation of collagen compared to other proteins. These data support the hypothesis that after a large accumulation of analog-containing collagen is maintained by cells, the rate of collagen synthesis is decreased by delayed feed-back inhibition or some related mechanism (Lane *et al.*, 1971).

Many of the normal dermal fibroblasts showed Golgi complex and the granular reticulum to be unusually prominent, especially when compared to the dermal fibroblasts from the LACA-treated embryo. This suggests that the normal cells were actively secreting protein-rich material. Perhaps the electron dense material present in the cisternal profiles of granular reticulum represents a secretory product of these cells and in some way plays a role in the synthesis of bundles of collagen fibrils. Since the

distinct Golgi complexes and granular reticulum profiles were not observed in the fibroblasts from LACA-treated embryos, the rate of proline synthesis (collagenogenesis) presumably is decreased by delayed feed-back inhibition.

The findings of previous investigators have also helped to explain another observation on the dermis of the LACA-treated embryo, e.g., lack of abundance of aligned fibrils in the extracellular matrix. If the analog-containing collagen is in fact extruded at a decreased rate, thereby preventing the continued synthesis of new collagen molecules, then the abnormalities aforementioned may have resulted from the absence of collagen fibrils in the right place at the right time.

Hydroxyproline

The hydroxyproline analysis demonstrated that the administration of LACA to the chick embryo did interfere with the amount of hyxroxyproline found in the collagen of the embryonic chick skin. Most of the literature on collagen content of chick embryo skin is given by Levene and Gross (1959), Kivirikko (1963), and Woessner et al. (1967). Accumulation of collagen in the skin of normal embryos increases very rapidly between days 9-16. However, the hycroxyproline content of the embryos receiving single and triple injections of LACA decreases sharply from the amount found in the control. Kivirikko (1963) reported that there is a large rise in skin collagen from 9-16 days. His data also indicate a relatively sudden spurt in collagen concentration during this same age in the entire embryo. The large increase in collagen skin concentration revealed by a chemical assay agrees well with the electron microscopical results in the current study. At

11-12 days there was an increased accumulation of fibrils in the extracellular matrix. Within the next few days, these fibrils increased in quantity and formed large bundles.

The relatively low rate of hydroxyproline content in the skin from embryos at 6-8 days is misleading. It tends to create the impression that very little collagen is being formed during this period, when in fact, according to Woessner et al. (1967), rapid growth of the skin is taking place. And, as mentioned before, it is during this time that dermal condensations are beginning to form and the dermal pattern of papillae is laid down.

An interesting aspect of this analysis is that the differences in hydroxyproline content between the control single injected embryos and triple injected embryos was very small at days 15-16 and even smaller at days 6-8. This may mean, as mentioned earlier, that 11-12 days of incubation represents a very crucial stage for LACA susceptibility with respect to skin and feather morphogenesis and that treatments with LACA (disruption of collagenogenesis?) had a more distinct effect on feather growth and morphogenesis during this period than at 6-8 days or 13-16 days.

Experiments have been performed on characterizing the chemical and physical properties of the collagen molecule present in embryos treated with the proline analog LACA. Lane et al. (1971) isolated acid-soluble collagen from normal and LACA-treated embryos. The collagen from both embryos had the same molecular weight by gel filtration. The amino acid

composition of the isolated collagen was essentially the same except that it contained about 4 residues of LACA per 1000 residues of amino acids. The only demonstrable abnormality in the physical properties of the collagen from treated embryos was that the Tm value for the helix-coil transition was 1.8° lower and the early part of the melting curve was less sharp than the melting curve of the control. Since the Tm values of various collagens are directly correlated with their total content of proline and hydroxyproline, Takeuchi and Prockop (1969) suggested that the decrease in Tm may be explained by the fact that the introduction of about 4 residues of the analog into collagen decreased the proline and hydroxyproline content by an amount which was large enough to change the thermal stability of the molecule. An alternate explanation for the change in the melting curve is that substitution of LACA for proline and hydroxyproline may have extended critical bond angles in the polypeptide chain (Fowden and Richmond, 1963) and thereby made the triple helix less stable. Thus, this report supports present findings by explaining why the abnormalities that were present in the LACA-treated embryos were apparent. If the thermal stability of the collagen molecule has been altered and the triple helix less stable, then it is likely that the physical abnormalities would cause a problem for the extrusion of the analog-containing collagen molecule.

In conclusion, the use of a proline analog such as LACA, in an *in vivo* study where the action of the analog is specific and in many cases predictable, provides a very useful probe for diagnosing specific

morphogenetic and biochemical occurrences as they relate to the acquisition of the determined state and to the expression of that state through phenotypic differentiation. The conclusion is warranted that feather morphogenesis is indeed dependent on collagenogenesis both for epidermal-dermal interactions and for the subsequent expression of this interaction through differentiation of mesenchymal and epithelial cells.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. Single injections (0.9 cc; 1 mg of LACA/cc of saline) of L-azetidine-2-carboxylic acid (LACA) were given to experimental embryos at pre-incubation, 16-18 hr, 24 hr or 48 hr of incubation.
2. Triple injections (0.6 cc/injection; 1 mg of LACA/cc of saline) were given to experimental chicken embryos for three consecutive days, pre-incubation, 24 hr, and 48 hr of incubation.
3. The injections of LACA caused several gross abnormalities, particularly suppression and/or inhibition of normal feather development.
4. The time at which the single injections of LACA were made proved to be critical. The highest percentage of feather suppression occurred in embryos that were injected at 16-18 hr of incubation. However, triple injections caused an even greater percentage of feather suppressions and/or inhibitions.
5. The embryos were observed in groups: Group I (6-8 days), Group II (9-10 days), Group III (11-12 days), Group IV (13-14 days), and Group V (15-16 days). The highest percentage of feather suppressions occurred in embryos at days 11-12.
6. Skin whole mounts showed that the skin of LACA-treated embryos did not always exhibit the normal pattern of feather distribution and that papillae were suppressed.

7. Light microscopical studies showed that (1) feathers of LACA-treated embryos were suppressed in their outgrowth and elongation process; (2) there was a decrease in the density of dermal condensations; (3) there was a lack of basement membrane formation at the epidermal-dermal junction; (4) inhibition of epidermal placodes, and (5) suppression of barb-vane ridge formation.
8. Scanning electron microscopy of skin from LACA-treated embryos also showed obvious suppressions and/or inhibitions of feathers along with (1) the lack of distinct extracellular fibers in the dermis, (2) unorganized arrangement of dermal tissue and (3) "epidermal blistering" of the peridermal sheath in apterylar regions.
9. The transmission electron microscopical analysis of skin of LACA-treated embryos revealed (1) decrease in the number of fibrils at the epidermal-dermal junction, (2) noticeable decrease in the number of fibrils secreted by the dermal fibroblast, (3) unorganized lattice arrangement of the collagen fibrils, and (4) some distinct differences in the cytoplasmic integrity when compared to the control.
10. The hydroxyproline analysis of skin from embryos treated with LACA indicated that the hydroxyproline content of these embryos decreased upon increased concentration of azetidine. This indicated that the proline analog was probably incorporated into the collagen molecule.
11. It is apparent from these studies that LACA causes malformations in the skin of chick embryos, possibly by interfering with the normal rate of synthesis and extrusion of the collagen molecule. The exact mechanisms by which the feather suppression occurred is not

exactly known, but the proposal is offered that the proline analog altered the normal structure of the collagen molecule which subsequently decreased the rate of collagen extrusion and thus decreased its synthesis through negative feed-back inhibition, thereby causing suppression and/or inhibition of normal skin and feather development.

LITERATURE CITED

Aydellote, M. B. and D. M. Kochhar (1972) Development of mouse limb buds in organ culture: chondrogenesis in the presence of a proline analog, L-azetidine-2-carboxylic acid. Devel. Biol. 28:191-201.

Becker, U. (1976) N-H_2-Terminal extensions on skin collagen from sheep with a genetic defect in conversion of procollagen into collagen. Biochemistry, 15:2853-2862.

Berg, R. A. and D. J. Prockop (1973) The thermal transition of a non-hydroxylated form of collagen: evidence for a role for hydroxyproline in stabilizing the triple-helix of collagen. Biochem. Biophys. Res. Commun., 52:115.

Bernfield, M. R. (1970) Collagen synthesis during epitheliomesenchymal interactions. Devel. Biol. 22:213-231.

Blobel, G. (1977) Synthesis and segregation of secretory proteins: the signal hypothesis. In International Cell Biology. B. R. Brinnley and K. R. Porter, eds., pp. 318-325, Rockefeller University Press, New York.

Bloom, W. and D. W. Fawcett (1968) A Textbook of Histology. W. B. Saunders Co., Philadelphia.

Borstein, P. (1974) The biosynthesis of collagen. Ann. Rev. Biochem. 43:567-603.

Brothman, H. F. (1977) Abnormal morphogenesis of feather structures and pattern in the chick embryo integument. II. Histological description. J. Exp. Zool. 200:107-112.

Cairns, J.M. and J.W. Saunders (1954) The influence of embryonic mesoderm on the regional specification of epidermal derivatives in the chick. J. Exp. Zool. 127:221-284.

Cardinate, G.J. and S. Udenfriend (1974) Prolyl hydroxylase. Adv. Enzymol. 41:245-300.

Coulombre, A.J. and J.L. Coulombre (1972) Corneal development. IV. Interruption of collagen excretion into the primary stroma of the cornea with L-azetidine-2-carboxylic. Devel. Biol. 28:183-190.

Diegelmann, R.F. and B. Peterkofsky (1972) Collagen biosynthesis during connective tissue development in chick embryo. Devel. Biol. 28:443-453.

Ede, D.A., J.R. Hinchliffe, and H.C. Mees (1971) Feather morphogenesis and feather pattern in normal and talpid mutant chick embryos. J. Embryol. Exp. Morph. 28:65-83.

Eyre, D.R. (1980) Collagen: molecular diversity in the body's protein scaffold. Science, 207:1315-1322.

Fessler, J.H. and L.I. Fessler (1978) Biosynthesis of procollagen. Ann. Rev. Biochem. 47:129-162.

Fowden, L. and J. Richmond (1963) Replacement of proline by azetidine-2-carboxylic acid during biosynthesis of proline. Biochim. Biophys. Acta, 71:459-461.

Galbraith, D.B. and E.J. Kollar (1974) Effects of L-azetidine-2-carboxylic, a proline analog, on the in vitro development of mouse tooth germs. Arch. Oral Biol. 19:1171-1176.

Grant, M. E. and D. J. Prockop (1972) The biosynthesis of collagen. N. Engl. J. Med. 286:194-199.

Grobstein, C. and J. Cohen (1965) Collagenase: effect on the morphogenesis of embryonic salivary epithelium in vitro. Science, 150:626-628.

Gross, J. (1973) Collagen. In The Chemical Basis of Life. P. C. Hanawalt and R. H. Haynes, eds., pp. 67-75. W. H. Freeman and Company, San Francisco.

Graunbaum, B. W. and D. Glick (1956) Studies in histochemistry. XLV. Determination of hydroxyproline in micrograph amounts of tissue. Arch. Biochem. Biophys. 65:260-267.

Guzman, N. A., F. J. Rojas and K. R. Cutroneo (1976) Collagen lysyl hydroxylation occurs within the cisternae of the rough endoplasmic reticulum. Arch. Biochem. Biophys. 172:449-456.

Hall, B. K. (1977) Thallium-induced achondroplasia in chicken embryos and the concept of critical periods during development. Teratol. 15:1-16.

Humason, G. L. (1968) Animal Tissue Techniques. W. H. Freeman Co., San Francisco.

Kallman, F. and C. Grobstein (1965) Source of collagen at epithelio-mesenchymal interfaces during inductive interactions. Devel. Biol. 11:169-183.

_____, J. Evans and N. K. Wessels (1976a) Normal epidermal basal cell behavior in the absence of basement membrane. J. Cell Biol. 32:231-236.

_____, J. Evans and N.K. Wessells (1967b) Anchor filament bundles in embryonic feather germs and skin. J. Cell Biol. 32:236-240.

Karnovsky, K.J. (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27:137A.

Kivirikko, K.I. (1963) Hydroxyproline containing fraction in normal and cortisone-treated chick embryos. Acta Physiol. Scand. suppl. 219:1-92.

_____ and L. Risteli (1976) Biosynthesis of collagen and its alterations in pathological states. Med. Biol. 54:159-186.

Koch, W. (1974) The effects of azetidine-2-carboxylic acid on embryonic mice. Anat. Rec. 178:393-394.

Lane, J.M., P. Dehm and D.J. Prockop (1971) Effect of proline analog azetidine-2-carboxylic acid on collagen synthesis in vivo. I. Arrest of collagen accumulation in growing chick embryos. Biochim. Biophys. Acta, 236:517-528.

_____, L.J. Parkes and D.J. Prockop (1971) Effect of the proline analog azetidine-2-carboxylic acid on collagen synthesis in vivo. II. Morphological and physical properties of collagen containing the analog. Biochim. Biophys. Acta, 236: 528-541.

_____, D.J. Prockop, P. Dehm and L.J. Parkes (1971) Inhibition of collagen synthesis in chick embryos by proline analog,

L-azetidine-2-carboxylic acid. J. Bone Jt. Surg. 53:1018.

_____, F.W. Bora, D.J. Prockop, R.B. Heppenstall and J. Black (1972) Inhibition of scar formation by the proline analog, cis-hydroxyproline. J. Surg. Res. 3:135-137.

Leach, A.A. (1960) Notes on a modification of the Newman and Logan method for the determination of hydroxyproline. Biochem. J. 74:7-71.

Lewis, A.M. and R. Hunter, Jr. (1978) Modification of feather morphogenesis in chicken embryos by L-azetidine-2-carboxylic acid. Amer. Zool. 18:131.

Lowenstein, W.R. and Y. Kang (1964) Studies on an epithelial (gland) cell junction. I. Modification of surface membrane permeability. J. Cell Biol. 22:565-571.

Levene, C.I. and J. Gross (1959) Alteration in state of molecular aggregation of collagen induced in chick embryos by B-amino-propionitrile (Lathyrus factor). J. Exp. Med. 110:771-789.

Maderson, P.F.A. (1965) The structure and development of squamate epidermis. In Biology of Skin and Hair Growth. P.F.A. Maderson, ed., pp 129-153. Elsevier, Amsterdam.

Miller, E.J. (1976) Biochemical characteristics and biological significance of collagen. Molec. Cell. Biochem. 13:165-192.

Mottet, N.K. and H.M. Jensen (1968) The differentiation of chick embryonic skin. Exp. Cell Res. 52:261-283.

Myllyla, R.H., H. Anttinen and L. Risteli (1977) Isolation of collagen glucosyltransferase as a homogeneous protein from

chick embryos. Biochim. Biophys, Acta, 480:113-121.

Neuman, R.E. and M.A. Logan (1950) The determination of hydroxyproline. J. Biol. Chem. 184:299-306.

Oikarinen, A., H. Anttinen and K.I. Kivirikko (1976a) Hydroxylation of lysine and glycosylation of hydroxylysine during collagen biosynthesis in isolated chick embryo cartilage cells. Biochem. J. 156:545-551.

_____, _____ and _____ (1976b) Effect of L-azetidine-2-carboxylic acid on glycosylations of collagen in chick embryo tendon cells. Biochem, J. 160:639-645.

Palmiter, R.D., J.M. Davidson and J. Gagnon (1979) N-H_2-Terminal sequence of the chick pro alpha (I) chain synthesized in the reticulocyte lysate system. J. Biol. Chem. 254:1433-1436.

Porter, K.R. and G.D. Pappas (1959) Collagen formation by fibroblasts of the chick embryo dermis. Biophys. Biochem. Cytol. 5:153-165.

_____ and K.B.M. Reid (1978) The biochemistry of the complement. Nature 275:699-704.

Prockop, D.J., R.A. Berg and K.I. kivirikko (1976) Intracellular steps in the biosynthesis of collagen. In Biochemistry of Collagen. G.N. Ramachandran, A.H. Reddi, eds., pp 163-273. Plenum Press, New York.

_____, K. Kivirikko, L. Tuderman and N.A. Guzman (1979) The Biosynthesis of collagen and its disorders. N. Engl. J. Med., 301:13-23.

Ramachandran, G.N. and A.H. Redi (1976) Biochemistry of Collagen. Plemum Press, New York.

Rawles, M.E. (1963) Tissue interactions in scale and feather development as studied in dermal-epidermal recombination. J. Embryol. Exp. Morphol., 11:765-789.

Reynolds, E.W. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell.Biol. 17:208-212.

Rosenberry, T.L. and J.M. Richardson (1977) Structure of 18S and 14S acetylcholinesterase: identification of collagen-like subunits that are linked by disulfide bonds to catalytic subunits. Biochemistry, 16:3550-3558.

Sengel, P. (1958) Reserches experimentales sur la differenciation des germes plumaires et du pigment de la peau del l'embryon de poulet in culture in vitro. Ann Sci. Nat. Zool. 11:431-514.

Sengel, P. (1964) The determination of the differentiation of the skin and the cutaneous appendages of the chick embryos. In the epidermis. W. Montagna and W.C. Lobitz, Jr., eds. Academic Press, New York.

Serafini-Fracassini, A. and J.W. Smith (1974) The structure and Biochemistry of Cartilage. Churchill Livingston, Edinburgh.

Sobel, M.E., T. Yamamoto and S.L. Adams (1978) Construction of a recombinant bacterial plasmid containing a chick pro alpha 2 collagen gene sequence. Proc. Natl. Acad. Sci.

USA, 75:5846-5850.

Strudel, G. (1975a) Effects of L-azetidine-2-carboxylic acid and of 5-bromodeoxyuridine, in vitro and in vivo, on the development of the chick skeleton. Proc. 4th Conf. Europ. Teratol. Soc. B10.

_____ (1975b) Control of the phenotype vetebral cartilage differentiation by the periaxial extracellular material. In Extracellular Matrix Influence on Gene Expression. H. Slavkin and R.C. Greulicj, eds., pp 665-670. Academic Press, New York.

Stuart, E.S. and A.A. Moscona (1967) Embryonic morphogenesis: role of fibrous lattice in the development of feathers and feather patterns. Science, 157:947-948.

Takeuchi, T. and D.J. Prockop (1969a) Biosynthesis of abnormal collagen with amino acid analogs. I. Incorporation of L-azetidine-2-carboxylic acid and cis-4-fluoro-l-proline into protocollagen and collagen. Biochim. Biophys. Acta, 175:142-155.

_____. (1969b) Biosynthesis of abnormal collagen with amino acid analogs. II. Inability of cartilage cells to extrude collagen polytides containing L-azetidine-2-carboxylic acid or cis-4-fluoro-l-proline. Biochim. Biophys. Acta, 175:156-164.

Uitto, J. and D.J. Prockop (1973) Rate of helix formation by intracellular procollagen and protocollagen: evidence for a role for disulfide bonds. Biochem. Biophys. Res. Commun.

55:904-911.

_____ and _____ (1974a) Biosynthesis of cartilage procollagen: influence of chain association and hydroxylation of prolyl residues on the folding of the polypeptide into the triple helical conformation. Biochemistry, 13:4586-4591.

_____ and _____ (1974b) Incorporation of proline analogs into collagen polypeptides: effects on the production of extracellular procollagen and on the stability of the triple-helical structure of the molecule. Biochim. Biophys. Acta, 336:234-251.

Weiss, P. (1961) The biological foundation of wound repair, Harvey Lecture Ser., 55:13-42.

Wessells, N.K. (1962) Tissue interactions during skin histodifferentiation. Devel. Biol., 4:87-107.

_____ (1965) Morphology and proliferation during early feather development. Devel. Biol., 12:131-153.

_____ (1967a) Differentiation of epidermis and epidermal derivatives. N. Engl. J. Med., 277:21-33.

_____ (1967b) Problems in the analysis of determination, mitosis and differentiation. 18th Hahneman Symp., Philadelphia.

_____ and J.H. Cohen (1968) Effects of collagenase on developing epithelia in vitro: lung, ureteric bud, and pancreas. Devel. Biol., 18:294-309.

_____ and J. Evans (1968) The ultrastructure of oriented cells and extracellular materials between developing feathers.

Devel. Biol., 18:42-61.

_____ (1977) Tissue Interactions and Development. W.A. Benjamin, Inc., Menlo Park, Ga.

Moessner, J.F., R.I. Bashey and R.J. Boucek (1967) Collagen development in heart and skin of the chick embryo. Biochim. Biophys. Acta, 140:329-338.